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INHIBITION AND STIMULATION OF TUMOR HOMOIOTRANSPLANTS BY PRIOR INJECTIONS OF LYOPHILIZED TUMOR TISSUE [1]

By GEORGE D. SNELL, *research associate*, ARTHUR M. CLOUDMAN, *research associate*, ELIZABETH FAILOR, *research assistant*, and PATRICIA DOUGLASS, *research assistant*, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

INTRODUCTION

Following the discovery that the regression of a transplanted tumor produces a partial or complete immunity to a second transplant of the same tumor, there were numerous attempts to produce a similar immunity by the injection of nonliving tumor substance. While many of these attempts met with failure, there have now been reported a number of cases of definite success. Curiously enough, besides the cases in which immunity has been obtained, there are also cases in which the injection of tumor extracts prior to the inoculation of living tissue has resulted in a stimulation in the growth of the transplanted tumor.

The literature has been reviewed by Woglom (*1*) and Spencer (*2*). The following summary is confined to cases in which the injection of nonliving tumor substance has inhibited or stimulated the growth of subsequently inoculated living tumor, special emphasis being placed on papers published after the date of Woglom's review. The material is grouped by the animal used, the work with mice, rats, and rabbits being presented in that order.

There is one possible source of confusion in summarizing published results, namely, the fact that authors do not always distinguish clearly between tumor transplants that are entirely negative (negative transplants), transplants that grow and then regress (regressions), and transplants that grow progressively and kill the animal (positive transplants). Results will be summarized under these three headings when possible; when the author uses an ambiguous word such as "takes," the author's own language will be adopted.

STUDIES WITH MICE

Bridré (*3*) increased the percentage of mice negative to a tumor of rather low virulence by a single prior injection of tumor tissue dried over sulfuric acid and resuspended in saline. Fifteen of twenty test animals, and 11 of 24 controls were negative. Slightly more definite results were obtained by giving 1 to 3 prior injections of a tumor extract prepared by grinding tumor tissues, diluting slightly, and heating for 25 minutes at 55° C. The test inoculation was given 32 to 34 days after the first injection. All 14 test animals, and 9 of 20 controls were negative.

Koenigsfeld (*4*) prepared an extract by drying tissue from tumors IIIa and V in a vacuum apparatus, grinding, and resuspending in saline. Five series of 25 to 50 mice each, with controls, were

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given 4 to 8 injections, usually intraperitoneal, ranging from 2 to 35 mg. of dried tissue per injection, and were then inoculated with living tumor. All series showed poorer tumor growth in the injected animals, but the results were disturbed in most cases by a high mortality during the injections. In series IV, due apparently to a wider spacing of the injections (8 injections increasing from 2 to 35 mg. over a period of 56 days, with inoculation 5 days after the last injection), only 2 of 48 mice were lost. At 54 days after the inoculation, 31 of the 46 surviving test mice were dead, 10 were alive with large tumors, and 5 with small tumors. The controls, as usual, were all positive (10 inoculated, 10 dead).

Kepinow (*5*), using a tumor which usually gave 100-percent takes in white mice, reported immunity following the injection of an extract of boiled tissue. Treated mice received 3 intraperitoneal injections of 1 cc. each, of boiled tumor tissue stored prior to use in physiologic salt solution plus toluol, at 3-day intervals, and an inoculation of living tumor several days after the last injection. All 25 controls grew large tumors. In most of 25 test mice, the tumors were the size of a pea or smaller, in about 20 percent they were larger but never reached the size attained in the control.

Tizzoni and De Angelis (*6*, *7*, *8*) produced a partial immunity to Ehrlich's mouse carcinoma with a single prior injection of cells of the tumor killed with phenol or formol. Controls of the strain used were regularly killed by this tumor in 25 to 35 days. Of 26 mice injected with macerated tissue treated with 0.5 percent phenol (optimum duration of treatment, 24 hours), 7 survived subsequent inoculation of the tumor, and 13 others showed definite protection. Of 22 mice injected with macerated tissued treated with 3 parts of 2.5 percent formol in saline, 7 survived, and an additional 5 showed retarded tumor growth as compared with the controls. It was found that macerated tissues stored with formol in the refrigerator for 15 days gave maximum protection. Immunity was stated to be evident by 11 days after the immunizing injection and to persist for at least 4 months.

Fardon (*9*) found that 1 subcutaneous injection of 0.5 cc. of a filtered, cell-free embryo extract given 7 days prior to inoculation with mouse carcinoma 63 (strain of mouse not stated) reduced the percentage of takes from 97 for the controls (36 mice) to 57 for the test group (36 mice). For the same extract after 12 hours' storage in the ice chest, the percentages were: controls, 97 (36 mice); and test, 75 (36 mice). A protein fraction prepared by precipitation with 50 percent alcohol gave the following: controls, 57 percent (36 mice); test, 95 percent (36 mice); and test in repeat experiment, 75 percent (36 mice). Thus an enhancing rather than an inhibiting effect was apparently produced.

Domagk and Hackmann (*10*) used an extract of Ehrlich's mouse carcinoma prepared by grinding in 10 parts of water, centrifuging for one-half hour at 3,000 r. p. m., and filtering through an "Entkeimungsfilter." In some cases, the tissue was frozen and thawed before grinding. The sterile, cell-free extract was freshly prepared for each injection. In a typical experiment, 6 albino mice were given 5 subcutaneous injections ranging from 0.1 to 0.6 cc. at intervals of 1 to several days and were inoculated 10 days after the last injection. At 38 days, 5 of the mice were negative, and 1 had a tumor. Ten controls all had tumors. With fewer injections, the inhibition was less pronounced. The immunity was shown to persist for at least 3 weeks after the last injection. A test of the effect of storage indicated that the extract remained effective after 1 day at 0° C. but was partly inactivated after 8 days at 18° or 1 day at 37°. Three injections of the albuminous precipitate obtained by passing carbon dioxide through the cell-free extract largely inhibited the growth of subsequently inoculated tumors; the supernatant similarly injected produced little effect in one experiment but caused inhibition in a second. Extracts of nor-

mal mouse liver and lung proved active, though less so than the tumor extract. Both normal and tumor extracts, but particularly the latter, produced stimulation of tumor growth when injected in large doses into mice already carrying a transplant.

Dittmar (*11*) repeated Domagk and Hackmann's experiments with confirmatory results and added some evidence that the inhibitory effect was specific. Prior injection of extract of Jensen's rat sarcoma, and in one case of extract of a mouse sarcoma, failed to inhibit the growth of subsequently inoculated Ehrlich's carcinoma.

MacDowell et al. (*12*) used strain C58 mice, and the fraction of line I leukemic cells which was sedimented with a high-speed centrifuge (prepared by Dr. Claude; see Claude (*13*)). Of 20 mice given 1 injection 10 days prior to inoculation with the tumor, 1 survived; of 20 mice given 3 injections, the last 10 days prior to inoculation, 7 survived. All controls, 20 and 19, respectively, died, as is invariably the case with this tumor. This experiment is noteworthy in two respects: (1) It is the only experiment of this type so far described in which inbred mice were used; and (2) it is unusual among tumor-immunity experiments in that the leukemia employed originated in the strain immunized (strain C58). In regard to this second point, however, it should be noted that strain C58 can be immunized to line I leukemia by repeated injections of living cells, provided the first doses are very small (*14*).

Haaland (*15*) found that mice injected with a fluid prepared from mouse carcinoma 63 by grinding the tumor cells for 1½ hours at the temperature of liquid air, or in a mortar packed in ice and salt, showed not immunity, but increased sensitivity to subsequent inoculation of the living tumor. Since the frozen and ground inoculum produced no tumor, it was presumed to be free from living cells. The inoculation of living tissue was made 10 to 30 days following 1 injection of this material in doses ranging from 0.025 to 0.5 cc. In 1 experiment, all 10 test mice showed tumors, the mean weight of which at about 32 days was 3.37 gm.; 8 of 10 control mice showed tumors, the mean weight of which was 2.03 gm. Similar results were obtained in a number of other experiments. A fluid extracted from carcinoma 63 with a tissue press also produced hypersensitivity.

Leitch (*16*), using carcinoma 63, but a strain of mice somewhat more resistant than that employed by Haaland, obtained similar but more pronounced results. Leitch's extract for injection was prepared by grinding the tumor with sand, shaking in saline, storing in a cold chamber for 2 days, decanting the supernatant, and storing at the freezing point until used.

Bisceglie (*17*) produced enhanced growth of Speyerhaus's mouse adenocarcinoma by giving four subcutaneous injections of 0.25 cc. each on alternate days, of a cell-free, Berkefeld-Kerze filtrate of the tumor 10 days prior to inoculation with the living tissue. In a typical experiment, five treated mice were all dead with large tumors 30 days after inoculation, while the controls still had small tumors. The extract also stimulated growth of the tumor in vitro.

Favilli (*18*) found that a series of 14–20 injections of 0.5 cc. each of a 5-percent solution of sodium caseinate (Kalbaum), all or part of them given before tumor inoculation, stimulated the growth of Ehrlich's mouse carcinoma.

Bertolotto (*19*), using three injections of 3 mg. each, of nucleoproteins prepared from mouse carcinomas and from the normal and cancerous tissues of other species by successive extraction with 0.25 percent bicarbonate of soda followed by precipitation with acetic acid, produced increased growth of Ehrlich's mouse carcinoma inoculated 2 days after the last injection. The effect though consistent was slight, and the difference between the test and control animals tended to disappear after the tenth or twelfth day. In a small experi-

ment with the nucleoprotein β (Bolaffi), an opposite effect was observed.

Casey (*20, 21*) prepared an "enhancing material" or "XYZ" substance by anaerobically refrigerating tumor tissue 14-60 days at 24° F., mincing and grinding, and emulsifying in 10 parts of saline. Mice were given 1 subcutaneous injection of 0.1 to 0.5 cc. and were inoculated 12-18 days later. As indicated by percent takes and mean volume of tumors at 20-28 days, Bashford tumor XYZ substance enhanced the growth of the Bashford tumor in R. I. or Swiss mice (mean volume in tests, 3.2 cc.; in controls, 1.8 cc.), and sarcoma-180 substance enhanced the growth of sarcoma 180 (tests, 1.42 cc.; controls, 0.43 cc.). The effect was specific; no stimulation and in some cases possibly slight inhibition were produced by Brown-Pearce rabbit-tumor extract on Bashford tumor, by 180 extract on mouse sarcoma 37 and mouse carcinoma 63, and by carcinoma-63 extract on carcinoma 48.

Putnoky and Hary (*22*), using a strain of Ehrlich's mouse carcinoma carried for many generations in rats, found evidence of enhanced growth of the tumor in rats, which 10 days prior to inoculation had been given a single subcutaneous injection of a tumor emulsion prepared by grinding in saline and incubating for 1 hour at 56° C. on 2 successive days. Incubation at 70° or 100° seemed to reduce the effect.

STUDIES WITH RATS

Apparently the first studies of a tumor-stimulating substance were made by Flexner and Jobling with the rat (*23–25*). These authors found that the number of positive transplants of a rat sarcoma was increased if the rats were given a prior injection of tumor cells, in saline, heated for ½ hour at 56° C. Two or three injections seemed to be more effective than one. The effect persisted without marked decrease for 20 or 30 days after the last injection.

Chambers and Scott (*26, 27*) found an enhancing effect of an extract of Jensen's rat sarcoma on the growth of the tumor. In a typical experiment, tumor tissue was ground, washed repeatedly with N saline and then with N/20 hydrochloric acid in saline, and the solid residue was suspended in N/20 hydrochloric acid. This was incubated for 3 hours at 37° C., neutralized, and centrifuged, and the clear supernatant was used for injection. Eighteen rats were given 1 cc. each subcutaneously and inoculated with living tumor 7 days later, together with 24 controls. The tumors averaged 17.3 cc. at 21 days in the test animals, and 6.7 cc. in the controls. Other experiments showed that heating the fluid did not destroy its stimulating properties and that a potent extract could be obtained when the incubation at 37° was carried out for 18 hours in the absence of oxygen.

Scott and co-workers also studied an inhibiting extract obtained from the Jensen rat sarcoma. There is some doubt as to the significance of their earlier experiments (*28*) in that the extracts used, although aged and otherwise treated to the point where they produce no detectable tumors, were not cell-free. However, in a recent paper (*29*), they reported that the ooze squeezed with a clamp from a regressing tumor on a living rat under anesthesia, when injected subcutaneously, reduced the growth of subsequently inoculated living sarcoma cells. The ooze was squeezed through the sterilized skin and was cell-free. Nineteen test rats showed a mean tumor volume of 18.7 cc. as against 75.4 cc. for the controls. If the tumors from which the extract was obtained were growing instead of regressing, the extract was stimulating. Extracts from X-rayed tumors were inhibiting or without effect, according as the tumor was regressing or not regressing.

Sugiura and Benedict (*30*) reported immunity following injection of Sugiura rat-carcinoma cells heated to 45° for 30 minutes, but the evidence that this treatment kills all cells seems inconclusive.

STUDIES WITH RABBITS

Casey (*31–37*), in a series of studies with rabbits, showed that an "enhancing material" or "XYZ" substance could be obtained from the Brown-Pearce tumor by storing tumor tissue at 24° to 32° F. for 10 to 90 days and then grinding in saline. In some experiments, the tumor was embedded in paraffin during storage. Extracts of tissue stored up to 16 days would sometimes give rise to tumors, but longer storage apparently killed all cells. The active substance was shown to pass through a Berkefeld V filter, one intratesticular injection of the filtrate given 2 weeks prior to inoculation of living Brown-Pearce tumor cells causing a 64-percent increase in volume of the test tumors as compared with the volume in the controls, and a higher percentage of positive animals. Desiccated material was stated also to be active, but extracts of fresh tumor either lacked the enhancing substance or contained in addition some other substance which counteracted it. (No data were given on these last two points.)

Dmochowski (*38*), using serums of two rabbits injected with unheated extract of Brown-Pearce tumor and of two others injected with boiled extract of the tumor, found complement fixation in combination with the two extracts. While all four serums reacted alike with respect to the boiled and unboiled tumor extracts, they could be differentiated by their behavior in combination with boiled and unboiled extracts of rabbit pus.

Kidd and co-workers (*39-41*) studied by complement-fixation methods certain antigens present in the Brown-Pearce carcinoma and other rabbit tumors. In most cases the serums employed were produced by the regression of tumor transplants, but in 1 group of experiments complement-fixing serums were produced by the injection of tumor extracts. The Brown-Pearce tissue, previously stored at −22° C., often for several months, was ground in 9 volumes of dilute phosphate buffer, centrifuged at 4,400 r. p. m., the supernatant centrifuged again at 4,400 r. p. m., and the final supernatant used for injection. The extract thus prepared was free from visible cells and did not give rise to tumors. In a few cases a third centrifugation at 25,000 r. p. m. was added, the sediment being used in these cases. "Bluecross" rabbits were given 3 or 4 intraperitoneal injections over a period of 9 to 13 days and bled 7 or 8 days after the last injection. The serums were tested by complement fixation against Brown-Pearce and normal tissue extracts. Of 16 serums, 4 were positive with both types of extract, 5 with Brown-Pearce extracts only, 6 with neither, and 1 was negative with Brown-Pearce extract but gave a ± reaction with normal tissue. Four control serums were entirely negative. From these and other more extensive experiments, Kidd concluded that at least 2 types of induced antibodies are concerned: 1 that reacts with Brown-Pearce antigen only, and 1 that reacts with both Brown-Pearce- and normal-tissue antigens. The Brown-Pearce (and/or the normal-tissue antigen?) has been characterized by a variety of tests. It shows a large and nearly uniform particle size and weight, it is sedimented by centrifugation for 1 hour at 20,000 r. p. m., it does not pass through Berkefeld V and W candles or single Seitz disks, it passes through collodium membranes of 383 mμ average pore diameter but not of 348 mμ, it survives heating to 60° but not to 65° C., and it is rapidly inactivated by alcohol. The normal-tissue antigen is present in Brown-Pearce tumor tissue as well as in normal tissue and possesses certain physical characteristics in common with the Brown-Pearce antigen although distinct serologically.[2] The existence of a third antigen not sedimentable at 25,000 r. p. m. is suggested by the fact that serums of rabbits with the Kato sarcoma reacted with the supernatant fluid of several normal and neoplastic tissues, as well as with the sediment.

[2] Personal communication.

Kidd and co-workers found that strain differences are important in determining the capacity of rabbits to produce the various antibodies. Fifty Dutch, chinchilla, and agouti hybrid rabbits injected with cell-free saline extracts failed to produce the antibodies produced by 9 of 16 blue-cross rabbits as described previously. However, the serums of several agouti rabbits with growing Brown-Pearce tumors gave strong reactions with kidney and Brown-Pearce extracts. Different agouti and blue-cross rabbits reacted very differently.

Blue-cross rabbits which had developed the Brown-Pearce antibody in response to injections of the antigen were regularly negative to inoculations of the tumor. On the other hand, those rabbits that failed to develop the antibody following treatment showed varying degrees of growth, ranging from small masses that soon regressed to progressively growing and metastasizing tumors. The presence of the Brown-Pearce antibody did not inhibit the growth of the V2 carcinoma or of rabbit sarcoma I (*42*).

In vitro tests showed that if Brown-Pearce cells were mixed with positive serums and incubated for 2 or 3 hours before inoculation, growth was inhibited (*42, 43*).

While previously induced Brown-Pearce antibodies thus regularly inhibited the growth of the Brown-Pearce tumor, the growth of this and other tumors (V2 carcinoma and sarcoma I) in previously untreated rabbits showed little relation to the titer of complement-fixing antibodies developed following tumor inoculation. Thus rabbits not treated prior to receiving tumor implants sometimes regressed the tumor even though no antibodies developed, and in other cases grew the tumor despite the appearance of antibodies in high titer (*39*).

Jacobs and Houghton (*44*), in repeating some of Kidd's experiments, obtained negative or inconclusive results, but Kidd points out that these authors did not use blue-cross rabbits.

EXPERIMENTAL PROCEDURE

Tumors for injection were prepared by freezing-drying or lyophilization. The tumors were cut in small pieces, sealed in a freezing-drying apparatus, frozen with salt and ice at a temperature of −10° to −15° C., and dried overnight with a desiccant (Drierite) under vacuum. The tissue was ground in a mortar, further dried for a day or more in a desiccator under vacuum, weighed out into tubes, which were sealed, and stored in a refrigerator. In some cases the tubes were sealed in vacuum. For injection, the dried tissue was mixed in a mortar either with Locke's solution in the proportion 100 mg. tissue to 4.4 cc. solution, or with mineral oil and Locke's solution in the proportion 100 mg. tissue, 0.4 cc. mineral oil, and 2.0 cc. solution.

The tumors used were C1498, a C57 black subline 6 myeloid leukemia, which gives fair-sized subcutaneous masses when inoculated with a trocar in the axillary region; L946AII, a bone fibrosarcoma also of C57 black subline 6 origin; and 15091a, an A strain mammary carcinoma.

The strains of mice used were C57 brown subline A, C57 black subline 6, B alb C, an F_1 hybrid between a Swiss stock and the B alb C, and the C57 leaden. All strains except the Swiss are highly inbred.

Because immunologic experiments with mice have shown that antibody titer is increased by the use of numerous small injections of antigen (*45, 46*), mice receiving the lyophilized tumor tissues in Locke's solution were given intraperitoneal injections of 0.2 cc. on 3 consecutive days followed by 4 days' rest, this schedule being repeated until 21 injections had been given. In a few cases, the injection dose was doubled for the last 3 to 6 days. Mice receiving tissues in oil and Locke's solution were given 7 injections at weekly intervals, 4 or 5 of them being subcutaneous injections of 0.2 cc. of the oil-Locke's-tissue and the remainder (because the oil produced persistent swelling) intraperitoneal or subcutaneous injec-

tions of the same amount of dry tissue in Locke's solution alone. Controls for this series were each given 7 weekly subcutaneous injections of 0.04 cc. oil. All mice were between 7 and 10 weeks old at the time injections were started.

A small "healthy" piece of the same line of tumor previously injected was implanted by the trocar method in the right axilla 8 to 12 days after the last injection. Controls were inoculated at the same time and with pieces of the same fresh tumor.

Rigid cleanliness was maintained throughout in the preparation and handling of tumors and extracts, but no attempt was made to achieve complete asepsis.

Inoculated mice were examined twice a week; and where tumors had developed, their outlines were sketched. No instrumental measurements of the tumors were made, but all observations of tumor growth were made by only one of the authors so that such variation as might be introduced by different observers was ruled out.

Animals that regressed the tumor and those that were negative were reinoculated.

RESULTS

In the first group of experiments, 18 C57 browns, 18 C57 blacks, and 19 B alb C mice were injected with lyophilized A strain carcinoma 15091a in Locke's solution; 9 C57 blacks and 10 B alb C with lyophilized 15091a in oil and the solution; 20 Swiss × B alb C with lyophilized C57 black strain sarcoma L946AII in Locke's solution; 10 Swiss × B alb C with lyophilized L946AII in oil and the solution; and 18 C57 leadens with lyophilized C57 black strain myeloid leukemia C1498 in Locke's solution. (These figures do not include 7 mice that died during the course of the injections. Also excluded are 2 mice, 1 test and 1 control, that died, after tumor inoculation, from causes other than tumor growth.) The injections of C57 browns with 15091a and of C57 leadens with C1498 were repeated at a later date, 10 and 6 mice, respectively, being used. The results of inoculating these injected animals and of comparable groups of controls, each with living tumor of the same line used for injections, are shown in table 1 and figures 1 to 4.

TABLE 1.—*Injected and control mice in which tumor transplants were negative, regressed, or positive*

Experiment No.	Tumor	Stock	Injections		Negative	Regressions			Positive	Total mice
			Number	Medium		Grade 1	Grade 2	Grade 3		
					Number	*Number*	*Number*	*Number*	*Number*	*Number*
1:										
Test	Strain A carcinoma 15091a.	C57 brown	21	Locke's solution	0	0	0	0	18	18
Control			0		0	8	10	2	0	20
2:										
Test	do	do	21	Locke's solution	0	0	0	0	10	10
Control			0		0	0	2	4	1	7
3:										
Test	do	C57 black	21	Locke's solution	0	0	0	0	18	18
			7	Oil and Locke's soln	0	0	1	1	7	9
Control			0		4	11	0	7	1	23
			7	Oil only	0	3	6	1	0	10
4:										
Test	do	B alb C	21	Locke's solution	2	5	1	0	11	19
			7	Oil and Locke's soln	0	2	0	0	8	10
Control			0		7	11	1	0	1	20
			7	Oil only	5	5	0	0	0	10
5:										
Test	C57 black strain sarcoma L946-AII.	Swiss × B alb C	21	Locke's solution	4	5	1	0	10	20
			7	Oil and Locke's soln	0	2	1	1	6	10
Control			0		0	0	2	13	5	20
			7	Oil only	0	0	4	3	1	8
6:										
Test	C57 black strain myeloid leukemia C1498.	C57 leaden	21	Locke's solution	12	5	1	0	0	18
Control			0		0	0	6	13	1	20
7:										
Test	do	do	21	Locke's solution	1	5	0	0	0	6
Control			0		0	2	2	2	0	6

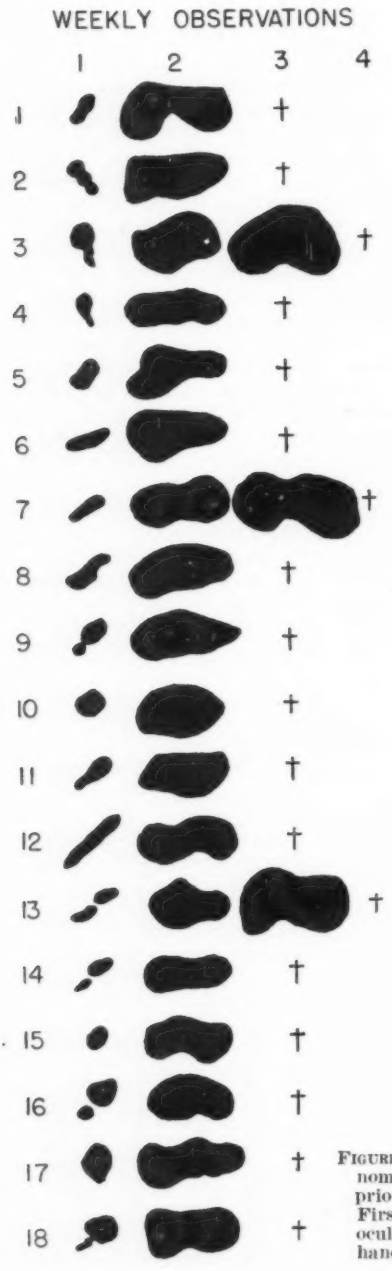

FIGURE 1.—Development of A strain carcinoma 15091a in C57 brown mice given 21 prior injections of lyophilized 15091a. First observation 1 week after tumor inoculation. Growth of the tumor is enhanced.

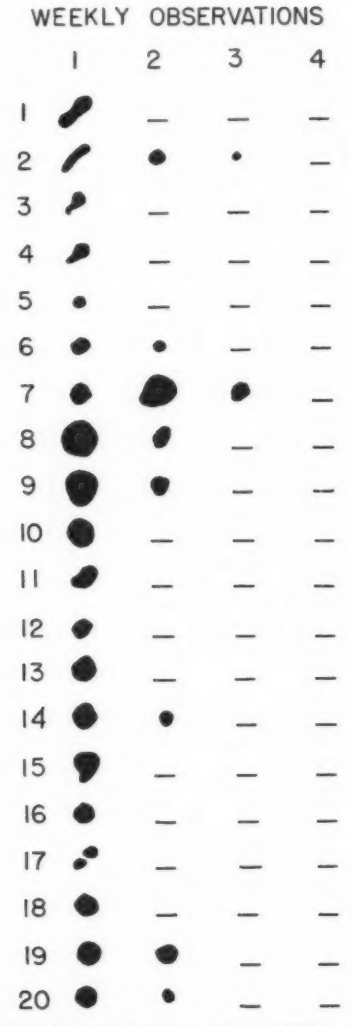

FIGURE 2.—Development of A strain carcinoma 15091a in untreated C57 brown mice; control to tumors shown in figure 1.

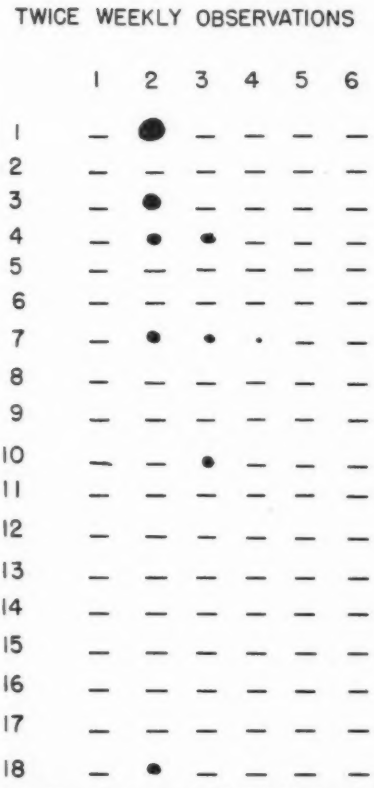

FIGURE 3.—Development of C57 black strain myeloid leukemia C1498 in C57 leaden mice given 21 prior injections of lyophilized C1498. First observation 4 days after tumor inoculation. Growth of the tumor is inhibited.

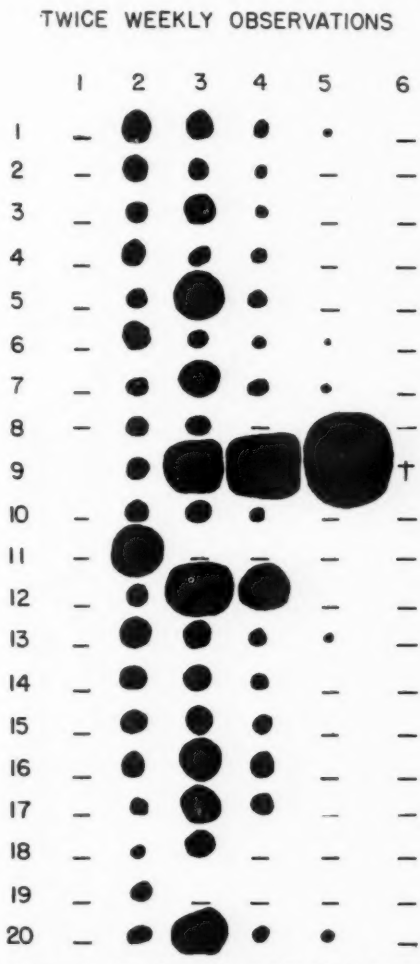

FIGURE 4.—Development of C57 black strain myeloid leukemia C1498 in untreated C57 leaden mice; control to tumors shown in figure 3.

In table 1 the regressions are classed under three hearings according to the maximum size attained as indicated by the outline sketches made at semiweekly intervals. Grade 1 includes tumors of not more than 5 mm. average diameter, grade 2 those of not more than 8 mm. average diameter, and grade 3 all larger tumors which ultimately regressed.

The prior injection of strain A carcinoma 15091a produced in all cases a very pronounced stimulation of tumor growth. In the C57 browns, 100 percent of the test animals were positive (killed by the tumor), and only 3.7 percent of the controls were positive. For the C57 blacks, the figures are 92.7 and 3.0 percent, and for the B alb C, 65.5 and 3.3 percent, respectively.

There was a slight stimulation produced by prior injection of lyophilized

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C57 black sarcoma L946AII. In this case, 53.4 percent of the (Swiss × B alb C)F_1 test mice and 21.4 percent of the controls were positive.

The prior injection of lyophilized strain C57 black leukemia C1498 produced an inhibition. Thus 54.1 percent of the C57 leaden test animals were negative (no growth) as compared with 0 percent of the controls. More significant is the maximum size attained by the tumors before regression (figs. 3 and 4). When the size of each tumor was measured by the average of the largest and the smallest diameter of the outline drawing made at the point of maximum development, the mean maximum tumor diameter for the test animals was found to be 1.6 mm., and for the controls 13.4 mm. The difference is more than eight times the standard error of the difference and hence undoubtedly significant.

The two systems of injection used (21 injections of lyophilized tumor in Locke's solution and 7 injections of lyophilized tumor in oil and Locke's solution) gave closely similar results.

Experiments 2 and 7, which were repetitions of experiments 1 and 6, respectively, indicate that the results are reproducible. It should be noted, however, that in experiment 7, the repetition of injections of strain C57 black leukemia C1498, the mean maximum tumor diameter is 3.8 and 11.2 mm. for test and control, respectively, as compared with 1.6 and 13.4 mm. for the original experiment. The reduced effect in the test animals of the second experiment is possibly significant, and if so may mean that minor fluctuations in the technique will have an influence on the results obtained in future experiments.

The results of reinoculating surviving animals of experiments 4, 5, and 7 are shown in table 2. Fifty-four out of a total of sixty-four reinoculations were negative or gave regressions of grade 1. However, in the case of experiment 4, four test and five control reinoculations were positive and one test reinoculation yielded a regression of grade 3.

TABLE 2.—*Repeat tumor inoculation*

Experiment No.	Previous inoculations	Negative	Regressions			Positive
			Grade 1	Grade 2	Grade 3	
		Number	*Number*	*Number*	*Number*	*Number*
4:						
Test	Negative	1	1	0	0	6
	Regressions	0	1	0	1	4
Control	Negative	4	2	0	0	1
	Regressions	2	6	0	0	4
5:						
Test	Negative	2	2	0	0	6
	Regressions	5	1	0	0	6
Control	Regressions	11	4	0	0	0
7:						
Test	Negative	0	1	0	0	6
	Regressions	4	1	0	0	0
Control	Regressions	4	2	0	0	0

DISCUSSION

The authors would like to emphasize at the start that they do not regard experiments in tumor immunity as having any but the most remote bearing on problems of cancer prevention or cancer therapy. This is because tumor immunity is essentially a reaction to foreign tissue, and a spontaneous tumor is not a foreign tissue. While in the case of one highly specialized cell, namely, the spermatozoon (*46*), animals can form antibodies against their own proteins, there is no satisfactory evidence that this has any parallel in tumor phenomena. The three cases (*12*, *47*, *48*) known to the writers in which immunity was produced in an adequately inbred strain (30–40 or more generations of brother-to-sister matings) are probably to be explained as the result of differentiation of either tumor or stock due to mutation. Haldane (*49*) (see also Russell (*50*)) states: "* * * we may expect that as a result of mutation most members of a mammalian pure line will be heterozygous for at least one gene." It necessarily follows that any pure stock is slowly but consistently undergoing minor changes in its genetic constitution. It is noteworthy that in two of the investigations cited, the tumor employed originated many transplant generations prior to the demon-

stration that it could evoke immunity in the strain of origin.[3]

While studies in tumor immunity are not likely to lead to results of immediate clinical value, they are of great theoretical interest in connection with the general problem of tissue specificity. There is also good reason to believe that they may serve as a research tool in studies of cellular chemistry. (See for example Kidd (*40*) and Friedenwald and Kidd (*39*).) It is in this light that the authors prefer to view them.

Perhaps the most striking fact brought out by a review of the literature on the induction of immunity by the injection of killed tissues is the diversity of the methods and the results. This is most clearly seen and best established in the case of studies with the Brown-Pearce and other rabbit tumors. Casey (*20*, *21*, *32–37*) was able to prepare an "enhancing material" from the Brown-Pearce tumor. Kidd on the other hand found evidence for the existence of Brown-Pearce- and normal-tissue antigens which would evoke complement-fixing antibodies when injected into some rabbits, but not into others. The Kato sarcoma probably contained a third antigen with quite different characteristics. The titer of the corresponding antibodies showed little if any relation to the growth or failure to grow of the various transplanted tumors employed. Hence the phenomenon of tissue specificity in this case would seem to depend entirely or in part on a different substance or group of substances. Kidd's studies further serve to emphasize the variety of the results obtainable by using different strains of animals. Dmochowski (*38*) found evidence for an antigen in the Brown-Pearce tumor which, unlike those studied by Kidd, was not destroyed by boiling.

The work here reported shows for the first time that the difference between stimulation and inhibition of transplanted tumors following injection of nonliving tumor material may be, in some instances at least, solely a matter of the tumor and stocks employed. C57 black tumor C1498 in the C57 leaden stocks was inhibited, and A strain tumor 15091a in the C57 brown stock was stimulated, following identical prior treatment with tumor tissues identically prepared. On the other hand, both stimulating and inhibiting substances have been extracted from the Brown-Pearce tumor, and the unusual method of extraction employed by Scott yields stimulating or inhibiting substances accordingly as the tumor is growing or regressing.

It is worthy of note that the inhibiting substance present in C57 black leukemia C1498 cannot correspond to the mouse blood-group antigens I, II, or III distinguished by Gorer (*51–53*), since all these antigens are lacking in the C57 black stock in which leukemia C1498 originated. However, it may correspond to Gorer's antigen IV since this antigen is present in the C57 black stock.

The diversity of the results just noted is probably to be explained by the complexity of the genetic basis of tissue specificity. The results in this field have been reviewed by Bittner (*54*) and Little (*55*). These results do not permit a precise estimation of the number of loci involved. (The term "loci" is used rather than "genes" in order to rule out multiple alleles. Since at any given locus there are usually a number of possible mutant genes, the total number of genes is ordinarily several times greater than the number of loci.) However, there is reason to believe that the total number of loci certainly exceeds 10 and is probably less than 30, and the most probable estimate would

[3] Since the present article was written, the description of a fourth case has appeared (Apteckman, P. M., Louis, M. R., and King, K. D.: A method of producing in inbred albino rats a high percentage of immunity from tumors native in their strain. J. Immunology, 52: 77–85 (1946)). Another recent paper (Cavelti, P. A.: Autoantibodies in rheumatic fever. Proc. Soc. Exper. Biol. & Med., 60: 379–381 (1945)), reporting the probable presence of autoantibodies to human heart in human patients with acute rheumatic fever, makes somewhat more plausible the concept that true autoantibodies to a tumor can be produced. Nevertheless, the fact remains that, because of the inherent nature of the genetic situation, the existence of genetic differences between tumor and host seems the more probable explanation of these four cases of immunity in an inbred strain to a tumor originating in that strain.

appear to be in the neighborhood of 16 or 20. It is possible that some of the genes are minor genes in the sense that whereas a mouse homozygous for the recessive allele of a major gene would be practically 100-percent resistant to a tumor carrying the dominant allele, a mouse homozygous for the recessive allele of a minor gene would grow such a tumor in an appreciable proportion of cases. Evidence for those statements will be presented elsewhere. If this view is correct, it is apparent that the number of loci concerned is much greater than in the closely analogous case of the human blood groups where there seems to be clear evidence for mutations at only about 4 loci. It seems probable that these loci in mice act to induce an immune reaction to a transplanted tumor by producing some substance not produced by the particular allele carried by the host. There is no reason to suppose that the number of such substances exceeds the number of genes concerned. On the other hand, it may be less, in that a number of loci may "collaborate" to produce a single end product as they apparently do in the case of the hair pigments in mammals. Yet it would appear that both genetic theory and the results of immunity experiments point to a more complex chemical basis for the phenomenon of tissue specificity than seems generally to have been assumed.

If this view is correct, it would seem that a complete unraveling of this basis will be difficult or impossible if ordinary noninbred stocks of animals are used. Even the use of the inbred stocks at present available will not go very far towards helping to separate and distinguish the different genes involved and the substances which they synthesize. With this in mind, methods have been developed for producing special inbred stocks identical with standard stocks now available except that each stock will be differentiated by carrying a recessive allele of some one of the loci concerned with tissue specificity. When tumor-immunity experiments are carried out with such stocks, there will be a strong presumption that the inhibiting or stimulating effect produced by prior injection of lyophilized tissues, or tissue otherwise prepared from the appropriate tumor, is due to a single chemical substance associated with a known gene. The analysis of the chemical basis of tissue specificity should be thereby greatly simplified. The production of these stocks has been started. The genetic methods to be used will be described elsewhere. Pending their development, tumor-immunity experiments are being continued with the stocks at present available.

SUMMARY

Mice of the C57 brown, C57 black, or B alb C strains were given 21 intraperitoneal injections of lyophilized A strain carcinoma 15091a in Locke's solution over a period of 7 weeks and were then inoculated with the fresh tumor 8 to 12 days after the last injection. In such mice, the tumor usually grew progressively and killed the animals. In controls, the tumor usually gave only small masses which later regressed or failed to grow at all. Seven subcutaneous injections of lyophilized tumor tissue in Locke's solution and mineral oil over a period of 7 weeks, followed by inoculation of fresh tumor, gave similar results.

When F_1 mice from a cross of Swiss $\times$ B alb C were similarly treated with lyophilized C57 black strain sarcoma L946AII and then inoculated with fresh sarcoma L946AII, a similar though less pronounced enhancing effect was obtained.

When C57 leaden mice were given 21 intraperitoneal injections of lyophilized C57 black strain myeloid leukemia C1498 and then inoculated with the fresh tumor 8 to 12 days after the last injection, the percentage of negative animals was 54.1 as compared with 0 for the controls. This series therefore showed a marked inhibitory effect, the exact opposite of the preceding series.

The relation of the genetic theory of tumor transplantation to tumor-immunity experiments is discussed.

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NOTE ON THE ENZYMATIC ACTIVITY OF NORMAL AND NEOPLASTIC TISSUES OF THE RAT

By JESSE P. GREENSTEIN, *principal biochemist*, and FLORENCE M. LEUTHARDT, *junior biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The study of a wide variety of normal and of transplanted neoplastic tissues of the mouse revealed that the range of enzymatic activity among the former kind of tissues was very much greater than among the latter.[1] It appeared as if the tumors, regardless of etiology or histogenesis, approached a common type of tissue.

The present note is a compilation of analogous data on normal and transplanted neoplastic tissues of the rat. Some of the data on arginase, on the phosphatases, on the desaminases, on the dehydropeptidases, on BAA amidase (benzoylargininеamidase), on cytochrome oxidase, and on esterase have been taken from earlier papers.[2] The methods of determining the activity of each of the enzymes and the mode of representing the activity so determined are given in a previous paper.[3] The data are collected in table 1, which shows that, similar to the case of mouse tissues, the range in enzymatic activity among the normal tissues of the rat is considerably greater than among transplanted tumors of this species. This range is also consistent with the nearly identical sulfur distribution values among rat tumors as contrasted with the much wider range of these values among normal rat tissues.[4]

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[4] See second reference in footnote 1.

TABLE 1.—*Enzymatic activity in normal and neoplastic tissues of the rat* [1]

Tissues	Arginase	Catalase	Acid phosphatase	Alkaline phosphatase	Ribonucleodesaminase	Desoxyribonucleodesaminase	Dehydropeptidase I	Dehydropeptidase II	Exocystine desulfurase	BAA amidase	Cytochrome oxidase	Esterase
Normal:												
Liver	213	2.0	25	4	20	4	20	20	45	32	11.8	312
Kidney	60	1.0	95	1,500	80	60	22	20	16	12	14.0	70
Pancreas	4	.1	18	3	[2] 10	0	17	10	8	8	.....	1,600
Spleen	10	.4	42	21	96	84	20	0	0	50	4.3	82
Brain	3	.0	16	14	32	0	18	0	0	0	13.0	4
Muscle	8	.1	16	2	0	0	18	0	0	0	6.3	4
Neoplastic:												
Transplanted hepatoma 31	21	.0	52	542	50	36	20	1	0	56	[3] 3.1	104
Transplanted Jensen sarcoma	46	.0	22	44	52	0	19	0	0	43	[3] 1.6	83
Transplanted tumor 2226 [4]	48	.0	32	18	50	5	20	0	0	40	2.0	72

[1] Activity units of each enzyme described in previous publication. See text, footnote 2.
[2] Extracts of this tissue rapidly desaminate adenylic acid (yeast), guanine, guanosine, but do not affect adenine. The desamination of the susceptible substrates is 60–70 percent of the theoretical after 1 hour of incubation.
[3] According to Schneider and Potter (Cancer Research, 3: 353–358 (1943)), the cytochrome oxidase activity of the transplanted hepatoma 31 and the Jensen sarcoma is very nearly the same.
[4] Very anaplastic epithelial type of tumor, donated by Dr. H. P. Morris.

REACTIONS OF BIS (2-CHLOROETHYL) SULFIDE (MUSTARD GAS) AND SOME OF ITS DERIVATIVES WITH PROTEINS AND AMINO ACIDS[1]

By JONATHAN L. HARTWELL, *senior chemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The renewed prominence into which bis (2-chloroethyl) sulfide (I)[2] was brought by reason of the war prompted an investigation into the mechanism of action of this substance upon the animal organism. The present paper represents a part of this investigation. In view of the ample evidence that the vesicants, iodoacetic acid and iodoacetamide, react under biologic conditions with the sulfhydryl group of proteins (*2–7*) and amino acids (*8–10*), as well as with the amino group of amino acids (*10*) and other amines (*11*), that the reaction with the sulfhydryl group is much more rapid than with the amino group, and that reaction takes place through the halogen atom of the vesicant, the possibility that mustard gas might exhibit a similar behavior appeared to be worthy of investigation. It had already been shown (*12*) that (I) readily reacts with simple mercaptans at elevated temperatures. However, with respect to the amino-group reaction, although Cashmore and McCombie (*13*) and Lawson and Reid (*14*) obtained a reaction between (I) and glycine ester and although the latter investigators showed that bis (2-chloroethyl) sulfone (II)[3] would react with glycine and phenylalanine, (I) has never been shown to undergo a reaction with a free amino acid. The attempts to carry out the condensation of (I) with a free amino acid (glycine and phenylalanine (*14*)), however, were carried, out under conditions where destruction of the vesicant by hydrolysis would be rapid. It seemed possible, therefore, that interaction might occur under more favorable circumstances. That (I) has an effect on proteins under physiologic conditions was shown in the experiments of Berenblum and Wormall (*15*), who demonstrated that the antigenic properties of serum proteins were changed by treatment with (I). No analytical data were given, nor was there a surmise as to the nature of attachment to the protein, however.

The present study demonstrates that (I) readily reacts under approximately physiologic conditions with the sulfhydryl and (with limitations) the amino group of proteins and amino acids, that the reaction with basic amino acids is much more rapid than with other amino acids, and that the reaction with the sulfhydryl group proceeds more readily than with the basic amino group.

EXPERIMENTAL PROCEDURE[4,5]

PREPARATIONS

(I) was prepared by the action of thionyl chloride on thiodiglycol according to the method of Steinkopf et al. (*16*). The thiodiglycol was obtained in part from Eastman Kodak Co. and used without further purification, and in part by synthesis from ethylene chlorohydrin according to the directions of Clarke (*17*). A nearly colorless product was obtained, which boiled at 109°–112°/24 mm. and turned light yellow on standing.

Bis (2-chloroethyl) sulfoxide (III)[6] was prepared according to the method of Helfrich and Reid (*12*) by the oxidation of (I) with concentrated nitric acid. One recrystallization from hot water gave white crystals, m. p. 109.5°–110.2° corrected.

[1] Submitted to the Office of Scientific Research and Development, Washington, D. C., June 2, 1943, and released for publication January 28, 1946.
[2] Hereinafter referred to as (I).
[3] Hereinafter referred to as (II).
[4] Except as noted, analyses are by Arlington Laboratories, Fairfax, Va.
[5] All reactions with proteins and amino acids were carried out at room temperature.
[6] Hereinafter referred to as (III).

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(II) was prepared according to the method of Helfrich and Reid (*12*) by the further oxidation of (III) by chromic acid. After one crystallization from alcohol, it was obtained as white plates, m. p. 56.9°–57.4° corrected.

REACTIONS WITH THE SULFHYDRYL GROUP

Reaction Between (I) and Denatured Egg Albumin

A technical grade of egg albumin (5.0 gm.) was dissolved in 100 cc. of water. To the solution were added 4.8 gm. of disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 .12H_2O$), 0.26 gm. of potassium dihydrogen phosphate (KH_2PO_4), and 100 gm. of urea. The solution, which had a pH of 8.0, was divided into 2 equal parts in glass-stoppered flasks, and the air in each flask was replaced by a commercial grade of nitrogen. One solution served as the control. To the other flask was added 0.64 gm. of (I), and both flasks were mechanically shaken. In spot tests, the nitroprusside reaction for the sulfhydryl group, which was strong at the beginning, was much weaker after 2 hours, for both mixtures; but when a little sodium cyanide was added to samples of each in order to reduce any disulfide back to sulfhydryl, the control gave a much stronger test. This showed that while some oxidation of the sulfhydryl group had occurred in the flask containing the (I), a considerable amount of the sulfhydryl had reacted with the (I). At this time also, the reaction of the solution was about neutral, and the (I) had disappeared. To each flask was then added 10 mg. of sodium cyanide, and to the flask containing (I) were also added 2.4 gm. of $Na_2HPO_4 .12H_2O$, 0.13 gm. of KH_2PO_4, and 0.64 gm. additional of (I). After shaking for 16 hours, the (I) had disappeared, and the nitroprusside test was negative in the treated solution and faintly positive in the control. When sodium cyanide was added to the spot tests, the control was much stronger than the treated solution.

The solutions were dialyzed in cellophane sacs against running water. As the urea was removed, the solution treated with (I) gradually became more opaque, until at the end the product had separated into white curds. The control showed no separation. The treated solution was centrifuged and the product dried under reduced pressure. An equal volume of alcohol was added to the supernatant solution and gave only a light turbidity. To the control solution an equal volume of alcohol was added, and the product was centrifuged off and dried under reduced pressure. Yields were: treated, 1.87 gm.; control, 1.64 gm.

Analysis gave the following results: treated, sulfur, 3.07, 2.98; nitrogen, 12.44, 12.61. Similar data for the control were: sulfur, 2.31, 2.23; nitrogen, 12.93, 12.75. The analyses showed an increase in the sulfur: nitrogen ratio of 37 percent, which corresponds to a combination of the protein with 4 percent of its weight of (I).

Formol titration of the products showed a decrease of free amino groups in the treated sample of about 35 percent, while a Van Slyke amino nitrogen determination[7] indicated a decrease of about 70 percent in free amino groups. Polarographic analysis of the products for sulfhydryl-disulfide groups[8] showed a decrease of this constituent in the treated sample of about 20 percent, presumably all due to sulfhydryl since disulfides would not be expected to react with (I).

It is thus evident that both sulfhydryl and amino groups underwent reaction to produce a product notable for its insolubility in water.

Reaction Between (III) and Denatured Egg Albumin

An experiment similar to the preceding, but with (I) replaced by an equivalent amount of (III), was carried out. The strength of the nitroprusside test did not decrease more rapidly in the solution treated with (III) than in the control (loss of disulfhydryl because of incidental oxidation), and it was concluded that no appreciable reaction with (III) and the sulfhydryl groups had occurred.

Reaction Between (II) and Denatured Egg Albumin

An experiment similar to the one just preceding, but with the (III) replaced by an equivalent amount of (II) was made. The nitroprusside test became negative within 15 minutes. After dialysis, both treated and control samples remained in solution. When the amount of (II) was reduced to about one-sixteenth of the quantity previously employed, so that (II) and sulfhydryl were in about equimolecular ratio, the nitroprusside test became weak after half an hour. The results show the greater reactivity of (II) as compared with (I), although any conclusions based on rapidity of action of (I) and its sulfoxide or sulfone must imply a consideration of the lower solubility of (I) in aqueous solutions.

Reaction Between (I) and Cysteine[9]

Cysteine hydrochloride (1.0 gm.; 0.00634 mole) was dissolved in 14 cc. of water, and 1.06 gm. (0.0126 mole) of sodium bicarbonate was added. Then 1.01 gm (0.00634 mole) of (I) was introduced, and the whole mixture shaken, the pH of the mixture being maintained slightly alkaline to litmus by the addition of sodium hydroxide solution. Within

[7] [8] Carried out by Dr. Richard J. Winzler.

[9] Bacq (Bull. Soc. roy. sc., Liége, 11: 381 (1942)) reported the product of this reaction, but the work has been available to the present author only in abstract form (Chem. Zentralbl. 1: 749 (1943)) without details.

3 hours, a white crystalline precipitate had separated, and no (I) was visible. The nitroprusside test was strongly positive. An additional 1.01 gm. of (I) was added. After 2.5 hours, the nitroprusside test was weak, and after 18 hours, the test was very faint. When sodium cyanide was added to an aliquot, the sulfhydryl reaction was relatively weak, showing that some oxidation had taken place. The product was filtered, washed with water and alcohol, and dried. The yield was 0.95 gm.

The product was insoluble in boiling water or alcohol but was soluble in dilute hydrochloric acid and sodium hydroxide. Formol titration indicated the presence of one amino group for each carboxyl group and gave a molecular weight (for a dibasic acid) of 333. The calculated molecular weight for $S[CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH]_2$ is 328.5. The sodium salt crystallized from aqueous solution in glistening flat needles.

Reaction Between (III) and Cysteine

Cysteine hydrochloride (1.0 gm.; 0.00634 mole) was dissolved in 14 cc. of water. To the solution were added 1.06 gm. (0.0126 mole) of sodium bicarbonate and 1.11 gm. (0.00634 mole) of (III). After the mixture had been shaken for 5 days, the nitroprusside test was still strong and the (III) was not visibly reduced in quantity. It was presumed that there had been no appreciable reaction.

Reaction Between (II) and Cysteine

Cysteine hydrochloride (1.0 gm.; 0.00634 mole) was dissolved in 9 cc. of water. To the solution were added 1.06 gm. (0.0126 mole) of sodium bicarbonate and 1.21 gm. (0.00634 mole) of (II). After being shaken for 1.25 hours, the solution was thick with white crystals, and the nitroprusside test was much weaker than originally. Water (5 cc.) was added; and after 0.75 hour longer practically all the crystals of (II) had disappeared, and the nitroprusside test was weak. The product was filtered and washed with water until the cake gave a negative sulfhydryl test. The cake was extracted with boiling alcohol to remove any (II), and the product was crystallized from boiling water. An additional crop of crystals was obtained from the mother liquor. The total yield was 0.50 gm.

The product was sparingly soluble in cold water, soluble in hot water, soluble in dilute hydrochloric acid and sodium hydroxide, and insoluble in hot alcohol. Formol titration indicated the presence of one amino group for each carboxyl group, and gave a molecular weight (for a dibasic acid) of 357. The calculated molecular weight for $O_2S[CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH]_2$ is 360.5. The sodium salt was obtained as long white needles, by slow evaporation of the aqueous solution.

Reaction Between (I) and Glutathione

Glutathione (1.0 gm.; 0.00326 mole) was dissolved in 14 cc. of water, 0.55 gm. (0.00652 mole) of sodium bicarbonate was added, and the mixture shaken with 0.52 gm. (0.00326 mole) of (I). After 7 hours, the nitroprusside test was negative; and when sodium cyanide was added to a sample, the test was only weakly positive. After being shaken for 15.5 hours longer, the excess (I) had disappeared. The solution was exactly neutralized, and an equal volume of alcohol was added. A light-yellow, gummy material separated. This product was soluble in alcohol and in alcohol-ether and was never obtained in solid form.

Reaction Between (II) and Glutathione

Glutathione (1.0 gm.; 0.00326 mole) was dissolved in 14 cc. of water, 0.55 gm. (0.00352 mole) of sodium bicarbonate was added, and the whole shaken with 0.62 gm. (0.00326 mole) of (II). After 3 hours, the nitroprusside test was positive; but after 15.5 hours later, the test was negative even when sodium cyanide was added. To the neutral solution an equal volume of alcohol was added: a small amount of crystalline precipitate separated at −10° C. This was filtered off, and a larger second crop was obtained by adding an equal volume of alcohol to the filtrate. The yield was: first crop, 0.17 gm.; and second crop, 0.50 gm.

Formol titration of these products indicated the presence of the amino group and gave a molecular weight (for a dibasic acid) of 974 and 912, respectively. Since the calculated molecular weight of the free acid is 732.8, the products obtained were probably hydrated or contaminated with sodium chloride. Since the exact composition of these products was considered at this time to be of secondary importance to the fact that reactions involving the sulfhydryl group actually occurred, the products were not purified further. It is reasonable to assume, however, that glutathione resembles cysteine in its reaction with (I) and (II) and gives rise to the compounds $S(CH_2\text{-}CH_2\text{-}S\text{-}G)_2$ and $O_2S(CH_2\text{-}CH_2\text{-}S\text{-}G)_2$, respectively, where G represents the glutathione residue.

REACTION WITH THE AMINO GROUP

Reaction Between (I) and Denatured Egg Albumin

The reaction between (I) and denatured egg albumin has already been described in the previous section, and the evidence for involvement of the amino group presented.

Reaction Between (I) and Native Egg Albumin

A technical grade of egg albumin (5.0 gm.) was dissolved in 100 cc. of water to which 4.8 gm. of $Na_2HPO_4.12H_2O$ and 0.26 gm. of KH_2PO_4 were added. The solution, which had a pH of 7.4, was divided into two equal parts, of which one served as the control. The other part was shaken with 0.64 gm. of (I). Within 18 hours the excess (I) had disappeared. The solution

was made slightly alkaline to litmus with sodium hydroxide, another 0.64 gm. of (I) was added, and the mixture kept slightly alkaline by the frequent addition of sodium hydroxide. Within 24 hours the (I) had disappeared, and the solution treated with it was a little more turbid than the control. After dialysis against running water, the control solution contained no sediment whereas from the treated solution was deposited a large amount of amorphous material. This insoluble fraction (A) was centrifuged off and dried under reduced pressure. To the supernatant solution was added an equal volume of alcohol; the large amount of precipitate (B) obtained was centrifuged and dried in the same way as the insoluble fraction. The control was similarly precipitated and dried. The yields were: A, 0.42 gm.; B, 1.37 gm.; and control, 1.30 gm.

Analysis showed: A sulfur, 2.32, 2.49; nitrogen, 13.26, 13.51; B: sulfur, 2.37, 2.48; nitrogen, 13.65, 13.73; and control: sulfur, 1.49, 1.56; nitrogen, 14.11, 14.01. The analyses also showed an increase in the sulfur: nitrogen ratio of 66 percent for A and 63 percent for B, which corresponds to a combination of the protein with 5 percent of its weight of (I) in each treated sample.

Reaction Between (I) and Casein

Casein (5.0 gm.) was dissolved in 100 cc. of water with enough sodium hydroxide to make the solution slightly alkaline to litmus, and the solution was filtered and divided into two equal parts. One part served as a control; to the other was added 0.64 gm. of (I), and both solutions were shaken and maintained at slight alkalinity. At 2 hours, the solution had become thick with the precipitated product. The mixture was shaken for half a day longer and let stand several days, whereupon the curd consolidated into a small mass. The product (A) was made neutral to litmus, centrifuged, washed with alcohol, and dried under reduced pressure. An equal volume of alcohol failed to produce a precipitate in the supernatant solution. The yield was 0.29 gm.

A similar experiment was run in which the quantity of (I) was reduced to 0.32 gm. At 1 hour there was no visible excess of (I); at 2 hours, a sample taken for formol titration showed a reduction in the amino group of 36 percent; and at 7 hours, another sample showed a reduction of 38 percent. The solution, which showed no deposition of solid, was made neutral to litmus and precipitated with an equal volume of alcohol. The product (B) was centrifuged, washed with alcohol, and dried as in the other experiment. The yield of B was 0.81 gm., and that of the control 0.16 gm.

Analysis[10] gave the following: A: sulfur, 2.12, and nitrogen, 12.8; B: sulfur, 2.57, and nitrogen, 12.4; and the control: sulfur, 0.86,[11] and nitrogen, 14.2. The analyses showed an increase in the sulfur: nitrogen ratio of 173 percent for A and 242 percent for B. These increases correspond to a combination of the protein with 7 percent and 10 percent, respectively, of its weight of (I).

Reaction Between (I) and Amino Acids

dl-Alanine (1.0 gm.; 0.0112 mole) was dissolved in water, 0.94 gm. (0.0112 mole) of sodium bicarbonate was added, and the volume adjusted to 15.0 cc. The solution was shaken with 1.79 gm. (0.0112 mole) of (I) and tested occasionally to verify the slight alkalinity to litmus. A similar solution, omitting the (I), was used as a control. In order to follow the loss of the amino group, samples were removed from the solutions at intervals and submitted to formol titration.

It was observed (table 1) that the reaction of (I) with alanine was too slow to account for the ready reaction with proteins, only an 8-percent loss in amino group being observed after 2 days. With the expectation that basic amino groups might be more reactive, a similar series of reactions was run, using 1.0 gm. each of the basic amino acids *d*-arginine monohydrochloride,[12] *d*-lysine monohydrochloride, and *l*-histidine monohydrochloride, and using the same equivalent amounts of other reagents as for alanine, except for one additional equivalent of sodium bicarbonate to neutralize the hydrochloric acid in the amino acids. The results (table 1), calculated on the basis that only the basic amino group reacts, show a reactivity for the basic amino acids more consistent with the results obtained with proteins. Arginine and histidine reacted completely within 3 days, while lysine was less active, requiring 6 days for the reaction to be two-thirds complete although it was a little over 40 percent complete in 1 day. The fact that the controls showed essentially no loss of amino group even after 6 days indicates that the amino group in the treated solutions was actually entering into combination with the (I) and was not being lost by oxidation or some other process. Attempts to isolate the amino acid reaction products yielded no solid derivatives but only gummy masses, which resisted purification.

In carrying out the formol titrations, it was found necessary first to acidify the sample taken with hydrochloric acid in order to expel the carbon dioxide. The solution was then brought as usual to a faint pink color to phenolphthalein with sodium hydroxide, 5 cc. of neutralized formalin was added, and the solution titrated with 0.1000N sodium hydroxide. Only the last titration was recorded.

[10] Carried out in part by Mrs. Sylvia R. L. Kornberg.

[11] Value obtained from the literature.

[12] In the case of arginine (but not in the control) the solution was made up to 7.5 cc.

TABLE 1.—*Rate of reaction of some amino acids with bis (2-chloroethyl) sulfide at approximately physiologic conditions of temperature and hydrogen-ion concentration as measured by formol titration and expressed as percentage loss of the basic amino group* [1]

Amino acid	Sample	Time in days: 0 NaOH	0 Loss of NH_2	0.08 NaOH	0.08 Loss of NH_2	0.23 NaOH	0.23 Loss of HN_2	1 NaOH	1 Loss of NH_2	2 NaOH	2 Loss of NH_2	3 NaOH	3 Loss of NH_2	5 NaOH	5 Loss of NH_2	6 NaOH	6 Loss of NH_2	8 NaOH	8 Loss of NH_2
		Cc.	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*	*Cc.*	*Pct.*
dl-Alanine	0.90	5.60	0	5.35	4			5.15	8	5.16	8			3.60	36			3.32	41
dl-Alanine control	.45	2.74	0					2.79	[2] −4	2.86	−9					2.79	−4		
d-Arginine	.45	2.63	0			1.92	54	1.65	75			1.34	98	1.30	101				
d-Arginine control	.45	1.07	0					1.15	−15	1.04	6					1.11	−7		
d-Lysine	.45	3.03	0			2.64	26	2.40	42			2.36	44			2.01	67		
d-Lysine control	.45	2.84	0					2.87	−2			2.76	6			2.77	5		
l-Histidine	.90	1.58	0			1.25	42	1.06	66			.66	116			.68	114		
l-Histidine control	.90	1.58	0			1.45	[3] 16	1.36	[3] 28			1.58	0			1.56	3		

[1] Except *dl*-alanine, where the results are referred to the α-amino group.
[2] A negative percentage loss (i. e., an increase) in amino group is obviously impossible. It should be considered as a 0-percent loss within the limits of error.
[3] Figure probably in error since the results at 3 and 6 days show a negligible loss in amino group.

TOXICITY OF DERIVATIVES

The local and systemic toxicities in animals, of the various derivatives with amino acids, were given in another report (*1*). In general, the derivatives were practically without effect on mice, except the dicysteinyl derivatives of (I) and (II), which did exhibit toxic properties.

DISCUSSION

The results reported here extend to amino acids and proteins the observations of other workers on the condensation of bis (2-chloroethyl) sulfide (I) with mercaptans and amines. The sulfone (II) was found to be more reactive toward the sulfhydryl group than was (I), as had been reported (*12*, *13*) for the reaction of (I) and (II) with amines. The sulfoxide (III) showed a rather surprising lack of activity toward cysteine as it had toward glycine ester (*13*). The results reported here indicate a considerably greater reactivity of (I) and (II) with the sulfhydryl group than with the amino group of amino acids and proteins. This difference was also found for the vesicants, iodoacetic acid and iodoacetamide (*11*).

These results are not to be taken as confirmation of the "amino-acid theory" of the vesicant action of mustard gas (*13*), since it is evident that many other substances which react with amino and sulfhydryl groups are not vesicant. It is believed, however, that the results are consistent with the theory that mustard gas reacts chemically with constituents of the living cell. The knowledge that mustard gas is capable of combining with two groups which are almost universally present in living cells represents a basis from which further research may be projected. The action of mustard gas on the living animal is undoubtedly complicated. The fact that mustard gas readily combines with sulfhydryl groups suggests that enzymatic studies might yield interesting results. How great a part the change in physical properties of the proteins may play is entirely unknown. The present study also provides a background for further immunologic studies so promisingly begun by Berenblum and Wormall (*15*).

SUMMARY

Bis (2-chloroethyl) sulfide (I) combines, under approximately physiologic conditions of temperature and hydrogen-ion concentration, with sulfhydryl and amino groups in proteins. The products are characterized by

having lower solubilities. The sulfone (II) (but not the sulfoxide (III)) combines with sulfhydryl groups, at least in proteins.

The sulfide (I) and its sulfone (II) (but not the sulfoxide (III)) combine with the sulfhydryl group of cysteine and glutathione, under similar conditions, to form crystalline derivatives of the type $S(CH_2\text{-}CH_2\text{-}S\text{-}R)_2$ and $O_2S(CH_2\text{-}CH_2\text{-}S\text{-}R)_2$.

(I) combines readily, under similar conditions, with the basic amino groups of the basic amino acids *d*-arginine, *d*-lysine, and *l*-histidine. It combines much less readily with the amino group of *dl*-alanine.

The reaction, under approximately physiologic conditions, of (I) with the sulfhydryl group of amino acids is more rapid than the reaction with either the α or the basic amino group of amino acids.

The derivatives of mustard gas with the various amino acids show for the most part much less toxicity toward mice than does mustard gas itself.

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LIVER NECROSIS AND THE INDUCTION OF CARBON TETRACHLORIDE HEPATOMAS IN STRAIN A MICE

By ALLEN B. ESCHENBRENNER, *surgeon*, and ELIZA MILLER, *assistant biologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The focal necrosis of liver cells that is produced with a single dose of carbon tetrachloride is followed by rapid proliferation of the remaining liver cells. This regenerative growth is transitory and ceases when the liver has been restored to its normal complement of functioning cells. With repeated administration of this chemical, continuous regeneration keeps pace with the repeated necrosis. Under certain conditions of chronic administration of carbon tetrachloride to mice, nodules of neoplastic liver cells appear; and with cessation of treatment, they continue to grow while regenerative activity of the liver ceases.

The question naturally arises to what degree, if any, are the repeated necrosis and the chronic regenerative state related to the eventual appearance of hepatomas. Is carbon tetrachloride a carcinogenic agent in the sense of effecting specific changes in the liver cells that are manifested by the appearance of properties which we classify as neoplastic? If true, it should be possible to obtain tumors with nonnecrotizing doses. Or is carbon tetrachloride a nonspecific necrotizing agent, which when given repeatedly and producing cyclic necrosis and repair allows for the progressive selection of particular cell types having neoplastic potentialities? Or does carbon tetrachloride act as a carcinogenic agent only in the presence of necrotic liver tissue? If true, tumors would not be obtained with the use of nonnecrotizing doses, no matter over how long a period the doses are given. Or does carbon tetrachloride have specific carcinogenic properties for liver cells, and in addition, is the speed of the neoplastic transformation facilitated by selection of cell types through repeated necrosis and repair? If true again, theoretically it should be possible to induce these tumors with nonnecrotizing doses of this agent.

These questions led to a series of experiments which seem to indicate that repeated liver necrosis is not necessary for the induction of hepatomas in strain A mice with carbon tetrachloride.

MATERIALS AND METHODS

The animals used were strain A mice from colonies maintained at the National Cancer Institute. The mice, which were 3 months of age at the beginning of all experiments, were fed Purina dog-chow pellets and water ad libitum. The environmental temperature was maintained at 78° F.

The solutions of carbon tetrachloride in olive oil were administered by stomach tube in accordance with body weight, which was determined at the time of each feeding. The carbon tetrachloride was of chemically pure quality and was diluted with U. S. P. olive oil. Solutions were prepared by serial twofold dilutions of the most concentrated solution (32 percent by volume) and were stored at 42° F. when not in use.

Animals were killed by cervical dislocation. For histologic study, tissues were routinely fixed in Zenker-formol, dehydrated in ethyl alcohol, embedded in paraffin, and stained with hematoxylin and eosin. For determination of percent of liver cells rendered necrotic under different conditions of treatment, Wright's stain, N Wr 11,[1] buffered at pH 6, was used because of its sharp differentiation of the cytoplasm of necrotic and nonnecrotic liver cells. The procedure employed for this stain was that of Lillie (*1*). This involves no differentiation of individual sections, but instead their immersion in the various solutions for a constant period.

[1] This lot is essentially a mixed methylene blue and azure B eosinate and closely resembles Maximow's azure II eosin.

The duration of exposure to each solution used in dehydration, clearing, infiltration, and embedding of all tissues was held constant by the use of a commercial automatic tissue-changing device. Control of time of exposure and of the temperature of the paraffin was further facilitated by the use of a multiple tissue carrier and a multiple tray for embedding tissues, devised in this laboratory (*2*, *3*). This care was taken in order that the shrinkage incident to paraffin embedding be kept as uniform as possible for purposes of comparative histologic studies.

For the quantitative histologic measurements, the method recently devised by Chalkley (*4*) was used. Since it is a new method and the type of measurements obtained are different from those of other methods in common use, such differences are stressed. The values obtained by using this procedure are ratios expressed as percent of the area of a section that is composed of a particular tissue element (resting liver cells, liver cells in mitosis, necrotic liver cells) and do not represent numbers of cells or elements but areas of these elements in relation to each other or to the entire section. As briefly expressed by Chalkley (*5*, *footnote 1*, *p. 49*):

> This ratio is obtained by a method of sampling based on geometric probability and is done by repeated random presentation of a point pattern to the structures measured, and counting the visual contacts (hits) observed on the structures of interest.

EXPERIMENTAL PROCEDURES[2] AND RESULTS

Other experiments suggested to us that the minimum dose of carbon tetrachloride, which when given repeatedly will produce hepatomas, is probably not very different from the minimum liver-necrotizing dose. An attempt was made, therefore, to control every factor that might conceivably have an immeasurable influence on liver necrosis in relation to hepatoma induction.

A series of mice was killed at intervals following a single liver-necrotizing dose of carbon tetrachloride to determine the approximately optimal time for examination of the livers for the presence or absence of necrosis in relation to dose of the agent administered. The degree of liver necrosis produced with different doses of carbon tetrachloride, the minimum necrotizing dose, and the amount of animal variation in the inbred strains were determined. Accuracy of the interpretation of the results of these studies was enhanced by the use of Chalkley's method (*4*) for quantitative histologic analysis of the livers. Groups of mice of the same strain were fed in accordance with body weight. They received the same total amount of carbon tetrachloride over the same period, but with a variation in the number of doses into which the total amount was divided and hence a variation in the size of each dose. By using a graded series of doses therefore, it was possible to include a range in which one group received necrotizing doses while the other did not, the final total amount administered being the same.

NECROSIS AND REPAIR IN THE LIVER FOLLOWING A SINGLE DOSE OF CARBON TETRACHLORIDE

As a basis for experiments to determine the relation between doses of carbon tetrachloride and the amount of necrosis produced in the liver, a series of animals was killed at 12-hour inter-

[2] One method of approach considered was that of making biopsies of the livers during the course of chronic administration of the agent for the induction of tumors, to determine the presence or absence of liver necrosis. Following recovery from partial hepatectomy, however, there was previously noted an altered reactivity of the liver to subsequent administration of carbon tetrachloride (*6*) and also of chloroform (*7*). Although, to be sure, a biopsy is very small in comparison with the removal of 70 percent of the liver, as was done in these studies, we have no assurance that it would not have some effect on the organ. This objection, however, is not so serious as that of the effect of the laparotomy itself on the reaction of the liver to carbon tetrachloride, since it has been shown that subcutaneous inflammation is associated with an altered reactivity of the liver to this agent and to chloroform (*8*, *9*). Furthermore, a rather large number of biopsies would have to be carried out, which, besides being subject to possible variation in technique from one animal to another, could not be carried out on all animals at the same time in relation to the administration of carbon tetrachloride. There might be considerable fluctuation in body weight as a result of these procedures and consequently in the dose of the agent administered. This method was therefore discarded.

vals after a single dose of the agent. The mice were given 0.005 cc. of a 32-percent solution of carbon tetrachloride per gram body weight by stomach tube at 9 a. m. Two male and two female mice were killed at each 12-hour interval up to and including 5 days thereafter. Six hours prior to death by cervical dislocation, the mice were also given intraperitoneally 0.01 cc., per gram body weight, of a solution containing 5 mg. colchicine per 100 cc. saline. Sections of livers of all mice were dehydrated, embedded, sectioned, and stained as a group. Four sections were taken from different portions of each liver to obtain a somewhat representative picture of the entire organ.

A quantitative morphologic analysis of these sections, which were stained with azure eosinate buffered at pH 6, was made according to Chalkley's method. The ratios of the areas were determined for the following: (1) Resting liver cells; (2) liver cells in mitosis; (3) necrotic liver cells; and (4) other tissue (Kupffer cells, leukocytes, blood vessels, bile ducts, etc.). The percent of total liver-cell area rendered necrotic and the percent of nonnecrotic liver-cell area in mitoses were calculated for each animal (table 1). Average values for each time interval are shown in figure 1. No significant difference was found for these values

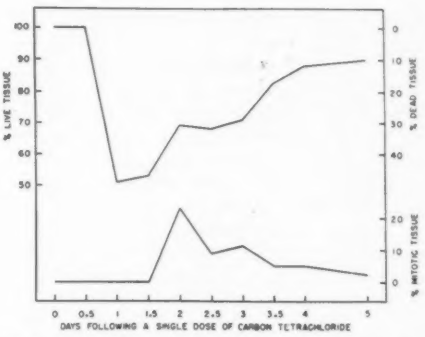

FIGURE 1.—Necrosis and repair in the livers of strain A mice at 12-hour intervals following an oral dose of carbon tetrachloride in olive oil. Each point on the curves is an average value for three or four mice.

in sections taken from different portions of a liver.

Twelve hours after a dose of carbon tetrachloride, there was a noticeable change in liver cells surrounding central veins. In sections stained with hematoxylin and eosin and with azure eosinate buffered at pH 6, the cytoplasm of these cells was slightly more acidophilic than was the cytoplasm of cells in the periphery of the lobules. Nuclei were intact. These areas around central veins approximately conformed in configuration and extent to the frankly necrotic areas seen 24 hours after the administration of the agent. The degree

TABLE 1.—*Necrosis and repair in the liver of strain A mice following a single dose of carbon tetrachloride* [1]

Sex of mice	Time in days																	
	½		1		1½		2		2½		3		3½		4		5	
	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis	Necrosis	Mitosis
Female	0	0	(2)	(2)	49	0	29	22	32	3	16	9	12	3	6	3	3	1
Do	0	0	46	0	44	0	(2)	(2)	28	13	31	8	6	1	21	5	23	7
Male	0	0	52	0	48	0	29	22	28	4	35	20	23	8	8	8	5	0
Do	0	0	49	0	49	0	35	25	41	16	34	10	29	10	13	5	8	1
Average	0	0	49	0	47	0	31	23	32	9	29	12	17	5	12	5	10	2

[1] Figures represent ratios, expressed as percent, of area of necrotic liver cells to total liver-cell area, and of area of mitotic liver cells to total nonnecrotic liver-cell area, in histologic sections. Time intervals are 12 or 24 hours. Colchicine was administered 6 hours prior to necropsy.

[2] Animals not included because of lymphocytic infiltration in portal areas of liver.

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of change in staining characteristics of these cells was not sharp in contrast with that of cells in the periphery of the lobules, and no cell was frankly necrotic. No attempt was made to determine the ratio of liver cells showing the slight differences of staining.

Twenty-four hours after a dose of carbon tetrachloride, there was a sharp demarcation between the central zones of necrotic liver cells and those immediately adjacent that escaped necrosis. This was particularly striking in sections stained with azure eosinate. Nuclei were absent or fragmented in the necrotic cells, and the cytoplasm stained a bright orange-red with coarse granulation, in contrast with the purple stain of the finely granular cytoplasm of nonnecrotic liver cells.

Thirty-six hours after a dose of the agent, the amount of necrotic liver-cell area relative to nonnecrotic liver-cell area was only slightly less than that at 24 hours. The apparent decrease in size of the necrotic regions if significant was probably due to a slight swelling of the remaining liver cells preparatory to division. Although a very occasional mitosis was encountered, the value for percent of cells in mitosis was far less than one.

Forty-eight hours after carbon tetrachloride administration, there was beginning infiltration of the necrotic areas with leukocytes; these areas were smaller, and 22 to 25 percent of the area of liver cells consisted of mitotic tissue. In preparations stained with azure eosinate, there was a noticeably more basophilic staining of the cytoplasm of all liver cells, which appeared as an intense, pure blue in contrast with the purple color seen in normal livers and in livers at intervals of 12, 24, and 36 hours after administration of the agent. Mitoses were scattered throughout the viable parts of the lobules but seemed more concentrated in the regions adjacent to the central necrotic areas.

At intervals after 48 hours and up to 5 days following the administration of the agent, a progressive decrease in size of the necrotic areas and a gradual decrease in mitotic activity were noted.

DEGREE OF LIVER NECROSIS IN RELATION TO THE AMOUNT OF CARBON TETRACHLORIDE ADMINISTERED

For purposes of the present study on tumor induction and in order to coordinate it with the findings in a previous report on the induction of these tumors (*10*), concentrations of carbon tetrachloride in olive oil ranging from 32 to ⅛ percent were tested. A single dose of 0.005 cc. of each solution per gram body weight was administered by stomach tube. Two male and two female mice were used for each dose. The animals were killed by cervical dislocation 24 hours after they had received the carbon tetrachloride. Sections for histologic study were prepared as previously described. Chalkley's method was used in analyzing section of the livers of all mice for the ratios of areas in three groups: (1) Nonnecrotic liver cells; (2) necrotic liver cells; and (3) other tissue components of liver. The percentage of liver-cell area rendered necrotic was determined for each animal (table 2).

TABLE 2.—*Area of liver cells of strain A mice rendered necrotic 24 hours after oral administration of various dilutions of carbon tetrachloride in olive oil* [1]

Sex of mice	Percent solutions of carbon tetrachloride								
	32	16	8	4	2	1	½	¼	⅛
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Female	51	46	47	46	41	42	29	10	0
Do	50	(2)	42	39	28	27	22	0	0
Male	48	49	46	45	37	48	38	0	0
Do	48	42	48	37	29	38	23	0	0
Average	49	46	46	42	34	39	28	....	....

[1] 0.005 cc. per gram of body weight.
[2] This animal not included because of lymphocytic infiltration in portal areas of liver.

In addition to the foregoing series of animals, another series was available that had been treated in the same way, but in the latter the livers were fixed in 95 percent alcohol for other studies to be reported later. Because of the different fixative, quantitative measure-

ments were not carried out, but the occurrence of necrosis in relation to dose of carbon tetrachloride was the same. One animal (a female) in four receiving the ¼-percent solution had necrosis.

The degree of necrosis in lobules throughout all lobes of the livers of animals receiving minimum necrotizing doses of carbon tetrachloride was approximately the same. This is in contrast with the observation (*11*) that minimum necrotizing doses of chloroform produce centro-lobular necrosis in only some lobes of the livers of strain A mice.

The possibility that two sub-necrotizing doses of carbon tetrachloride given 24 hours apart (as was done in the tumor-induction experiment described in the next section) might be additive and thus produce necrosis was considered. Groups of mice were therefore given two doses of the 1-, ½-, ¼-, and ⅛-percent solutions, respectively, 24 hours apart and were killed 24 hours after the second dose. These mice received 0.005 cc. of solution per gram body weight per dose. The doses were not additive. The degree of necrosis was not determined; only the presence or absence of necrosis was noted. The data are shown in table 3.

TABLE 3.—*Liver necrosis in strain A mice after administration of various dilutions of carbon tetrachloride in olive oil*

[Numerator = Number of animals showing liver necrosis
Denominator = Number of animals observed]

Sex of mice	Dose per gram body weight	Percent solution			
		1	½	¼	⅛
	Cubic centimeters				
Female	[1] 0.005	2/2	2/2	0/2	0/2
Male	[1] .005	2/2	2/2	0/2	0/2
Female	[2] .02	2/2	2/2	2/2	2/2
Male	[2] .02	2/2	2/2	2/2	2/2

[1] 2 daily doses.
[2] 1 dose.

In the tumor-induction experiment (see next section), one series of animals received 0.005 cc. of solution, while the other received 0.02 cc. of solution per gram body weight per dose. Since incomplete absorption of the carbon tetrachloride might occur in animals receiving the larger quantity of solution, groups of mice were given 0.02 cc. of the 1-, ½-, ¼-, and ⅛-percent solutions per gram body weight and were killed 24 hours later. Again, the degree of necrosis was not determined, only the presence or absence of necrosis was noted. These data are also given in table 3. Absorption appears to have been complete.

Another experiment on liver necrosis, done in connection with other work on carbon tetrachloride, is included (table 4) for its value in interpretation of the results of the tumor-induction experiment. In this experiment, a dose of various dilutions of carbon tetrachloride in olive oil, was administered to strain A mice, followed by a second dose 30 days later. Necropsies were made 24 hours after the second dose. Sections of livers of these mice were prepared, and quantitative histologic studies were carried out in the manner previously described. Each animal showed less liver necrosis than did the animals in the previous experiment, in which only a single dose of carbon tetrachloride was given. (See tables 2 and 4.) The necrotic areas were centro-lobular in distribution.

TABLE 4.—*Area of liver cells of strain A mice rendered necrotic 24 hours after a second dose of various dilutions of carbon tetrachloride in olive oil.*[1] *Compare with table 2*

Sex of mice	Percent solutions of carbon tetrachloride							
	32	16	8	4	2	1	½	¼
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Female	38	38	44	31	36	27	4	0
Male	38	32	36	33	38	35	0	0
Average	38	35	40	32	37	31	2	0

[1] First dose given 30 days previously.

HEPATOMA INDUCTION IN RELATION TO NECROSIS OF THE LIVER PRODUCED WITH CARBON TETRACHLORIDE

As mentioned in the Introduction, the plan of this experiment was to administer to two series of mice the same

total amount of carbon tetrachloride and olive oil over the same total period, but with a variation in the number of doses into which the total amount was divided and hence a variation in the size of each dose. Animals of 1 series were given 30 doses of carbon tetrachloride at intervals of 4 days while those of the other series were given 120 daily doses of the agent. The same solutions were used for the 2 series, mice of 1 series receiving 0.02 cc. of solution per gram body weight (30 doses) and those of the other series 0.005 cc. of solution per gram body weight (120 doses). A range of graded doses was used, which, from the data in the preceding section, when divided into 120 daily doses would include necrotizing and nonnecrotizing doses, but when divided into 30 doses given at 4-day intervals would include only necrotizing doses. Two control series of mice received 0.02 cc. and 0.005 cc., respectively, of olive oil per gram body weight per dose. Five male and five female mice were used in each of 7 different dose and 2 control groups. Unfortunately, the group of mice to receive 30 doses of the 1-percent solution was omitted in order to minimize the number of stomach-tube feedings. It was thought, contrary to the findings, that a higher incidence of tumors would follow 30 rather than 120 doses.

Treatment with carbon tetrachloride was started when the mice were 3 months of age and terminated when they were 7 months old; all animals were examined at 8 months of age for the occurrence of hepatomas. To throw further light on the question of tumor induction in relation to liver necrosis, all animals were given one additional dose of the solution 24 hours before necropsy. The results are shown in table 5. The data for probable initial necrosis in relation to dose of carbon tetrachloride are taken from tables 2 and 3.

Mice receiving the largest dose of the agent (1-percent solution) had numerous hepatomas up to 1 cm. in diameter; those receiving smaller doses (½- and ¼-percent solutions) had 2 or more smaller tumors. The hepatomas were larger and more numerous in groups of animals that received 120 doses than in those that received 30 doses of a given solution, in both groups the total amount of carbon tetrachloride given being the same. The presence or absence of hepatomas and of liver necrosis,

TABLE 5.—*Susceptibility of strain A mice to liver necrosis and the incidence of hepatomas 30 days after 120 or 30 doses of carbon tetrachloride* [1]

[Numerator = Number of mice showing necrosis or hepatoma
Denominator = Number of mice observed]

Sex	Dose conditions	Total carbon tetrachloride (per gram body weight)														
		60 x 10^{-4} cc. (1-percent solution)			30 x 10^{-4} cc. (½-percent solution)			15 x 10^{-4} cc. (¼-percent solution)			7.5 x 10^{-4} cc. (⅛-percent solution)			0 (olive oil)		
		Probable initial liver necrosis	Observed final liver necrosis	Hepatoma incidence	Probable initial liver necrosis	Observed final liver necrosis	Hepatoma incidence	Probable initial liver necrosis	Observed final liver necrosis	Hepatoma incidence	Probable initial liver necrosis	Observed final liver necrosis	Hepatoma incidence	Probable initial liver necrosis	Observed final liver necrosis	Hepatoma incidence
F	120 doses in 119 days	+	0/5	5/5	+	0/5	5/5	±	0/5	5/5	−	0/5	0/5	−	0/5	0/5
M	do	+	0/4	4/4	+	0/5	5/5	±	0/5	5/5	−	0/5	0/5	−	0/5	0/5
F	30 doses in 116 days				+	2/4	2/4	+	2/5	3/5	−	0/5	0/5	−	0/5	0/5
M	do				+	4/4	3/4	+	3/5	4/5	+	0/5	[2] 0/5	−	0/5	0/5

[1] Based on gross and microscopic examination.
[2] 2 animals showing no tumor on gross examination were found each to have a very small hepatoma on microscopic examination of a random section of the livers.

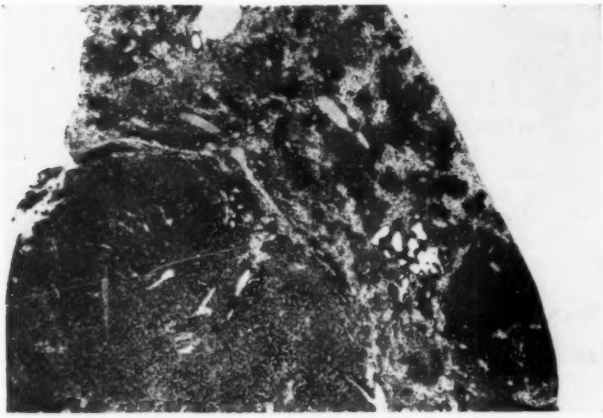

FIGURE 2.—Section of liver and hepatoma from a mouse that had received 30 doses of 1-percent solution of carbon tetrachloride at intervals of 4 days. Light areas are foci of necrotic liver cells. Note absence of necrosis in tumor. Azure eosinate buffered at pH 6. × 20.

as recorded in table 5, was established by histologic examination. No tumors were observed grossly in animals receiving the ⅛-percent solutions or olive oil alone. Random sections were made of livers containing no tumors grossly. By chance, a histologic section of liver of each of 2 mice receiving the ⅛-percent solution included a small nest of cells that appeared to be early hepatomas. Of the original 90 animals, 3 were lost during the experiment, possibly from perforation of the esophagus with the feeding tube, although this could not be established definitely. When areas of necrosis of the liver were observed in tumor-bearing animals, no necrosis was observed in the hepatomas (fig. 2). The localization of the areas of necrosis after chronic administration of carbon tetrachloride, however, seemed to follow no definite pattern (fig. 3, *B*) in contrast with the regular pattern of centro-lobular necrosis seen after a single dose (fig. 3, *A*). The areas of necrosis might surround or be adjacent to veins with or without associated bile ducts or might lie between the two (fig. 3, *B*). The normal approximate equidistance of central veins and of portal veins (as identified by associated bile ducts) was absent.

Sections of livers from four mice of each dose group (chosen at random) were stained with silver for demonstration of reticulum fibers. Representative sections of each dose group are shown in figures 4 and 5. A rough correlation was observed between the degree of distortion of the liver lobules and of the deposition of reticulum fibers and the amount of carbon tetrachloride administered. Further, there appeared to be no appreciable difference in the amount of reticulum in mice that received 120 daily doses as compared with those that received 30 doses at intervals of 4 days. The former were nonnecrotizing doses, and the latter were all necrotizing doses, while the total amount of a given solution that was administered was the same. Although the amount of reticulum in figure 5, *B* and *C*, seems slightly greater than that in figure 4, *B* and *C* (and these are representative sections), it would be hazardous to consider this slight difference significant in this experiment.

DISCUSSION

The quantitative histologic studies on necrosis and repair in the liver following carbon tetrachloride emphasize the value of Chalkley's new method. The

expenditure of 15 to 20 minutes' time per section establishes a figure that is reproducible, with an error of not more than 2 percent. In determining the effect of different doses and gaging the degree of variation between animals, therefore, it is not necessary to compare sections repeatedly in order to arrive at what can only be a rough approximation, or one which in the end may be expressed only as an opinion.

In the experiment on the relation between dose of carbon tetrachloride and amount of liver necrosis (table 2), animal variation was very slight with the larger doses and increased with dilution of dose. When, however, a large dose was used to study the course of necrosis and repair (table 1), and little animal variation was noted in the amount of initial liver necrosis, such variation became apparent as differences in rate of repair.

Minimum necrotizing doses of carbon tetrachloride were found to produce necrosis of lobules of all lobes of the liver while minimum necrotizing doses of chloroform were found in an earlier study (*11*) to produce necrosis of lobules of only some lobes of the organ. Copher and Dick (*12*) observed a "stream line" phenomenon in the portal vein of dogs, causing a selective distribution of portal blood in the liver from different portions of the gastrointestinal tract. A difference in the site of absorption of chloroform and of carbon tetrachloride would seem a more likely explanation for the observed differences in liver necrosis than that of a difference in susceptibility of liver cells in different lobes.

The use of colchicine facilitates the study of repair of the liver but at the same time introduces a complicating factor that should be kept in mind in any comparison with studies in which it is not used. Being toxic, it might thus have slightly different degrees of retardation of repair in livers at different stages of recovery from the necrosis produced by carbon tetrachloride. No histologic evidence of necrosis was seen in mice given colchicine without antecedent carbon tetrachloride. We can be reasonably sure, from the studies of Brues, Marble, and Jackson (*13*) on the effect of colchicine on regeneration of the rat liver after partial hepatectomy, that the dose we employed had no appreciable effect on the regeneration of the liver subsequent to carbon tetrachloride.

Mitoses were scattered throughout the lobules although they seemed to be more numerous near the areas of necrosis. This scattering of mitoses must mean that there is a migration of liver cells toward the central necrotic areas in addition to their appearing there as a result of division of liver cells at the very margin.

The possibility of a diurnal variation in rate of mitotic activity of the regenerating liver is indicated in figure 1 and table 1. All mice were fed the carbon tetrachloride at 9 a. m. and were killed either at 9 p. m. (12, 36, 60 hours, etc.) or at 9 a. m. (24, 48, 72 hours, etc.). While the slightly but consistently higher average rates seen in mice killed at 9 p. m. may not be statistically significant for the present series of animals, they suggest such a variation as a possibilty. This is in line with observations on diurnal variation of mitotic activity in mouse skin (*14*), in human skin (*15, 16*), in normal and regenerating rabbit skin (*17*), and in rat renal cortex (*18*). Of interest in this connection is the observation of mutually reciprocal relationship in the diurnal cycle of glycogen and bile salts in the liver (*19, 20*).

Our observations on the rate of repair of the liver of the mouse after carbon tetrachloride, as determined by quantitative histologic studies on the ratios of areas of necrotic, nonnecrotic, and mitotic tissue, compare favorably with those of Brues, Drury, and Brues (*21*) on the rate of repair of the liver in rats after partial hepatectomy. Their method consisted of following liver weights and of calculation of cell numbers, using average cell size and total liver weight. Since all the carbon tetrachloride is probably eliminated

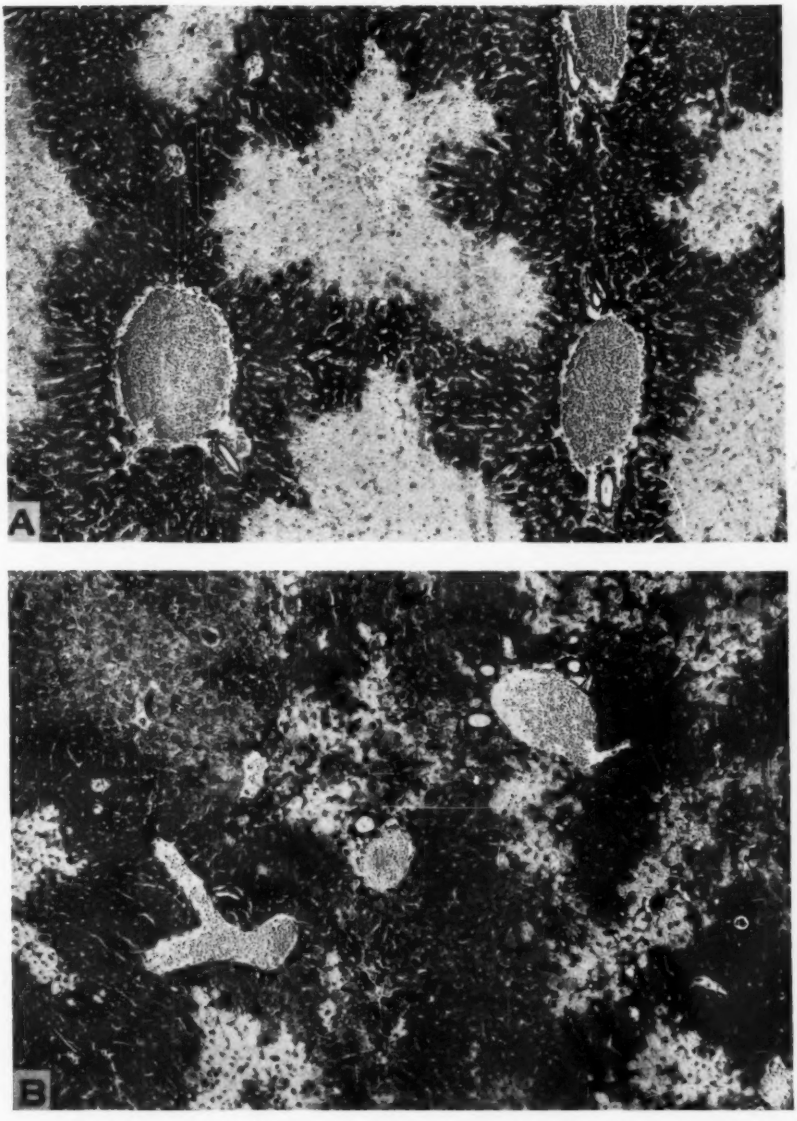

FIGURE 3.—Focal necrosis of the liver. *A*, Following a single dose of 1-percent solution of carbon tetrachloride; and *B*, after a period of chronic administration. Photographed with red filter (necrotic areas white). Azure eosinate buffered at pH 6. × 66.

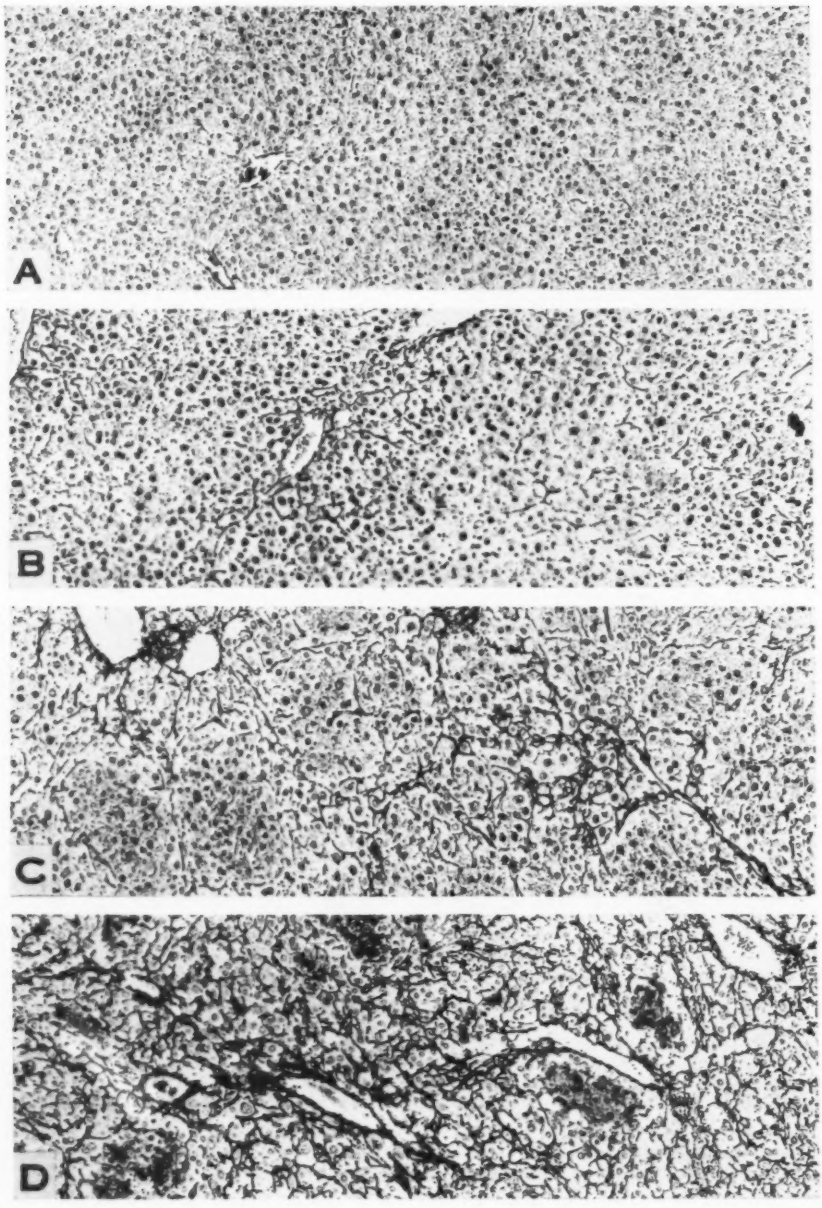

FIGURE 4.—Reticulum fibers in livers of mice receiving 120 daily doses: *A*, Of olive oil; *B*, ⅛-percent, *C*, ¼-percent, and *D*, ½-percent solution of carbon tetrachloride in olive oil. × 66.

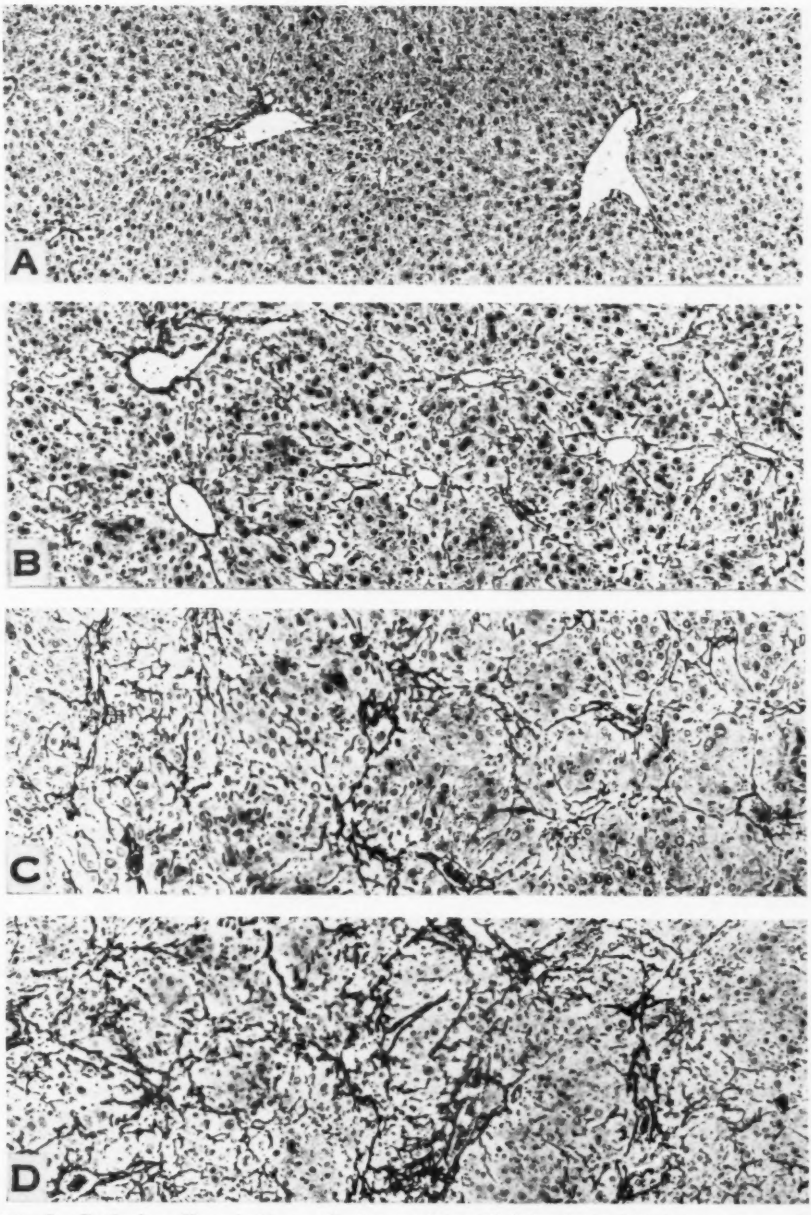

FIGURE 5.—Reticulum fibers in livers of mice receiving 30 daily doses, but 4 times as much per dose as the cases illustrated in figure 4. *A*, Of olive oil; *B*, ⅛-percent, *C*, ¼-percent, and *D*, ½-percent solution of carbon tetrachloride in olive oil. × 66.

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from the body within 24 hours (*22*), it is, in effect, similar to a partial hepatectomy in that in both instances there is a primary insult that results in the removal of a large proportion of liver cells.

A difference of time of onset of mitotic activity may be noted in comparing our studies with those of Brues and Marble (*23*) on the restoration of the rat liver subsequent to partial hepatectomy. Their quantitative studies demonstrated a maximum mitotic rate 24 hours after operation in contrast with our finding of a maximum 48 hours after administration of carbon tetrachloride to mice. They observed that mitoses were evenly distributed throughout each lobule, that the mitotic rate varied from hour to hour in the same animal, and that the fluctuations occurred at different times in different livers. These observations might throw some doubt on our assumption that our data indicate a difference of over-all rate of repair in different animals. We noted that in three mice the initial mitotic rate at 48 hours was very nearly the same, but that in other groups of four mice each at 12-hour intervals thereafter there was increasing variation in mitotic rate. Our studies are not strictly comparable with those of Brues and Marble in respect to the hourly fluctuation in mitotic rate, however, since our use of colchicine resulted in a mitotic index representing a 6-hour accumulation of mitoses, thereby obscuring such a phenomenon. Furthermore, the quantitative data on the relative size of areas of necrotic and nonnecrotic liver cells showed a trend similar to that of mitotic activity. At 24 and 36 hours before repair had begun, the areas of necrosis in seven animals were very nearly the same. With onset of mitotic activity, there was increasing variation in the size of the necrotic areas at successive 12-hour intervals in different animals. We can account for this only by assuming that it is a true reflection of variation in over-all rates of repair in different mice, following the same initial amount of liver necrosis. Such differences of over-all rates of repair, of course, may represent not only differences in the livers alone but also systemic differences in reaction to carbon tetrachloride.

A comparison of qualitative studies by Cameron and Karunaratne (*23*) on the course of necrosis and repair in the rat subsequent to carbon tetrachloride with those in the mouse presented herein indicate no appreciable species difference.

Necrosis of the liver produced with carbon tetrachloride can be regarded from two viewpoints in relation to hepatoma induction: (1) The term "necrosis" can be used only in reference to the massive necrosis found when the larger doses are used. With serial dilution of the dose, the amount of necrosis diminishes slightly and then is completely absent. (2) It can be postulated that the agent may have a delayed lethal effect on cells or may cause premature aging and death, with the necessity of replacement at a rate slightly greater than normal.

The two viewpoints necessitate different definitions of regeneration or hyperplasia that would necessarily follow the necrosis. There is no question but that the production of hepatomas with obviously necrotizing doses of carbon tetrachloride is associated with a chronic state of regeneration of the liver, although we know of no evidence to prove that a causal relation exists. On the other hand, it would be hard to prove or disprove that there is a slightly greater rate of replacement of liver cells without frankly necrotizing doses of the agent, and hence what might theoretically be considered to be a chronic regenerative or hyperplastic state, associated with the production of hepatomas with nonnecrotizing doses. This discussion is confined, therefore, to the role of obvious liver necrosis and its attendant regeneration in the genesis of these tumors.

One question that arises is whether or not we may assume that at least some of the 10 animals (each of which had tumors) given 120 doses of the ¼-per-

cent solution did not have repeated liver necrosis. We are dealing with a highly inbred strain of mice, to which the solutions were administered in accordance with body weight by stomach tube, the animals were of the same age, and after a single dose or 2 doses of the agent necrosis was observed in only 2 to 12 animals; the probability is therefore that this question can be answered in the affirmative. As another affirmative answer to the question whether hepatomas can be induced with nonnecrotizing doses of carbon tetrachloride, may be submitted the fact that no necrosis resulted from the dose given the day before necropsy. When a single dose of the agent was given, extensive necrosis was observed in all animals receiving the 1-, and ½-percent solutions, and in 2 of 12 animals receiving the ¼-percent solution. However, when 120 daily doses of these solutions had been given, followed by a rest period of 30 days, and an additional dose was given 24 hours before necropsy, none of 10 animals had liver necrosis. Thus, during the course of chronic administration of the agent, the livers of the animals developed a resistance or became insensitive to its necrotizing action. A comparison of tables 2 and 4 indicates that there is considerable resistance or insensitivity developed very early in the period of chronic feeding, beginning even as a result of the first of 120 doses, a fact that lends further weight to the probability that hepatomas can be induced with nonnecrotizing doses.

The question of the role of necrotizing doses in the induction of these tumors can be attacked in still another fashion by a comparison of the tumor incidence in relation to dose in mice receiving 120 doses with that of mice receiving 30 divided doses of the various solutions. Complete resistance or insensitivity to necrosis was developed in all mice that received 120 doses of each solution, whereas a comparable reaction was noted in some of the mice only that received 4 times as much carbon tetrachloride per dose for a total of 30 doses. If one takes the conservative attitude, the tumor incidence was no different; or if one considers the observed difference in tumor incidence to be significant, it was even lower in those that received more necrotizing doses. This fact suggests that other factors in dosage conditions are more important than the presence or absence of necrosis. A comparison of dose-response data for carbon tetrachloride and chloroform suggests that the carcinogenic properties of these agents are more closely related to those properties that cause liver necrosis than to the ones responsible for their anesthetic action. Under conditions similar to those of the present experiment, the minimum tumor-induction dose of chloroform was also the minimum necrotizing dose (*11*). For chloroform this dose was 4×10^{-4} cc. per gram body weight per dose, and for carbon tetrachloride between 1.0 and 0.5×10^{-4} cc. per gram body weight per dose. In each instance, 30 doses were given at 4-day intervals, with necropsy 30 days after the last dose. The narcotic activity of the two chemicals, however, is of the reverse order, a far smaller amount of chloroform than of carbon tetrachloride being required to produce a given depth of anesthesia (*25*).

The present data, together with the following data, emphasize the importance of the time over which a given total amount of carbon tetrachloride is administered, in the induction of hepatomas. Carbon tetrachloride remains in the body a short time, probably not longer than 24 to 30 hours (*22*), in contrast with many of the carcinogenic agents. Edwards and Dalton (*26*) observed no hepatomas in strain A mice when 0.12 cc. of carbon tetrachloride was administered in three divided doses in 1 or 2 weeks. On the other hand, these authors found that when mice were given 0.005 cc. of this agent 2 to 3 times per week for a total of 0.125 to 0.145 cc. (a comparable amount) over a space of 2 to 3 months, hepatomas were found in 70 percent of the animals. Subsequently, we (*10*) observed that when strain A mice were given 30 doses of a given size at intervals of 1 day, no

hepatomas were found 150 days after the first dose. Mice receiving 30 such doses at intervals of 2 or 3 days had a hepatoma incidence varying between 30 and 70 percent depending upon the size of the individual doses; whereas when 30 such doses were given at intervals of 4 or 5 days, nearly all mice had hepatomas 150 days after the first dose.

In addition to the factors of total dose and total time over which divided doses are administered, there is an additional factor of rate of intermittency of treatment (number of doses and interval between doses) that should be considered. Our previous experiments (*10*) using carbon tetrachloride suggested to us that intermittent treatment might be more effective than continuous exposure in the induction of neoplasia of the liver with this agent. The idea of intermittent treatment seemed attractive because of the course of necrosis and repair observed after a single dose. Thus, if the cyclic process of necrosis and repair were an important and necessary prerequisite for the induction of hepatomas with carbon tetrachloride, it would seem that a given dose would be more effective if given at a time after the previous dose when repair of the liver had begun. A consideration of the time of onset of repair following a single dose (fig. 1) and a comparison of the data on tumor induction after 120 daily doses and following 30 doses given at 4-day intervals mitigate against the importance of the factor of intermittent treatment under the conditions of these experiments.

An acquired relative resistance of the liver to various toxic agents has been reported under several conditions. Following their recovery from partial hepatectomy, rats showed a relative resistance to the hepatotoxic action of carbon tetrachloride (*6*) and of chloroform (*7*). A relative resistance of the liver to carbon tetrachloride also resulted from antecedent ligation of bile ducts in cats (*27*). After recovery from necrosis caused by uranium nitrate, the livers of dogs showed a relative resistance to that agent and to chloroform (*28*). The quantitative histologic studies (tables 2 and 4) on the resistance of liver to the hepatotoxic action of carbon tetrachloride 1 month after an initial dose are in accord with these reported observations. The finding that during the course of chronic feeding with carbon tetrachloride, the livers of mice develop a relative resistance to its lethal action (fig. 2) and that the hepatomas are completely resistant in this respect, suggests that the mechanism of neoplastic change here might be connected in some way with the mechanism of the development of such relative resistance. Yet these two phenomena, development of resistance and neoplastic change, may be coincidental bearing no causal relationship. For instance, the liver cells may develop a relative resistance by the acquisition of some new components and the tumors may become insensitive to the necrotizing action by loss of certain specific components that characterize normal liver cells. The latter appears possible in the light of Greenstein's enzyme studies on hepatomas and other neoplasms (*29*). It was felt unwise, therefore, to draw any conclusions.

In connection with the development of a relative resistance of the liver to the necrotizing action of carbon tetrachloride, the report of Lamson and Wing (*30*) on the effect of this agent on certain liver-function tests is of interest. They found an increase of bile pigments in the blood, a decrease of blood fibrin, a reduced tolerance to levulose, and a slowed clearance of phenoltetrachlorphthalein 48 hours after a single large dose of carbon tetrachloride. When the same dose was repeated every other day, these values returned to normal after about 2 weeks and remained normal, despite histologic evidence of repeated necrosis and the development of early cirrhosis.

In addition to the finding of a lessened degree of necrosis in relation to dose after chronic administration of carbon tetrachloride is the observation that the necrosis no longer follows a regular centro-lobular pattern. The liver lo-

bule is looked upon as the functional unit of the liver. Certain toxic substances (carbon tetrachloride and other chlorinated hydrocarbons, tannic acid, etc.) characteristically produce uniform necrosis in the center of the liver lobules while others (phosphorus, etc.) produce uniform necrosis of the periphery of the lobules. The localization of necrosis produced by a hepatotoxic agent might be considered to reflect a localization of different functions in different parts of the lobule, whether this is caused by differences in liver cells or differences in the blood supply that they receive, or both. Such a consideration would carry with it the implication that subsequent to chronic administration of carbon tetrachloride there is an architectural disorganization of the liver with respect to certain functions. Whether this is related to the development of hepatomas that are relatively devoid of the enzyme pattern that characterizes normal liver (*29*) is an interesting question, but pure speculation at present.

While we have not been concerned primarily with the problem of cirrhosis of the liver, certain observations indicate that recognizable, repetitious necrosis and repair may not be necessary for the production of cirrhosis of the liver with carbon tetrachloride; that is, provided that an increase of reticulum in the liver following small doses represents the same mechanism as do the increasing amounts of reticulum fibers and also of collagen, with distortion of the lobular architecture, which are found with increasingly larger doses. In the present studies, the livers were examined 1 month after the last dose of carbon tetrachloride. In all animals that had received 120 doses of the 1-, ½-, and ¼-percent solutions of carbon tetrachloride, reticulum stains revealed distortion of the lobules and large amounts of reticulum fibers. There is good reason to believe that animals receiving the ¼-percent solution did not suffer from repeated liver necrosis. In addition to this finding, however, fine reticulum fibers in excess of normal ones were consistently found scattered throughout the lobules of the livers of animals that had received 120 doses of the ⅛-percent solution of carbon tetrachloride. There was no distortion of the lobular architecture of the liver after the administration of this solution. We do not believe that these animals at any time received a dose of carbon tetrachloride large enough to produce any otherwise recognizable damaging effect on liver cells. None of the control animals that received 120 doses of olive oil had any increase of reticulum fibers over that in untreated mice.

Of further interest is the similarity of livers of mice that received 120 or 30 doses of a given solution. Mice given the latter dose at 4-day intervals received 4 times as much carbon tetrachloride per dose as did those given 120 daily doses. This difference in quantity of agent per dose also reflects a difference in the absence, presence, and degree of necrosis. These observations suggest that with intermittent exposure to carbon tetrachloride, the total amount administered and the total time involved are more important factors than is the size of individual doses (or the presence or absence of necrosis) in the production of cirrhosis of the liver as well as in the induction of hepatomas. Caution should be exercised, however, in attaching any significance to apparent correlations between cirrhosis and hepatoma induction, from the present data. An example of the need of such caution is in the case of cirrhosis and hepatoma induction in rats fed *p*-dimethylaminoazobenzene. Earlier studies with this compound indicated an apparent correlation in the occurrence of these two hepatic changes. Later studies by White and Edwards (*31*) showed that by dietary means cirrhosis could be diminished in incidence and degree, without an equal reduction in the incidence of hepatomas.

The question of the necessity of repeated necrosis of the liver in the induction of hepatomas with carbon tetrachloride might be settled more conclusively by using a larger series of animals or by feeding them over a longer period.

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Such an undertaking cannot be considered lightly since the present experiments involved over 7,000 stomach-tube feedings to mice. We have undertaken to throw further light on this question in another way. Edwards, Heston, and Dalton (*32*) induced hepatomas in strain L mice with carbon tetrachloride; we[3] have found that the necrosis range in relation to dose of the agent in this stram differs from that in strain A. The experiments presented herein are being repeated using strain L mice.

SUMMARY

Experiments are reported in which an attempt was made to determine the relationship between repeated liver necrosis and chronic regeneration and the induction of hepatomas in strain A mice with repeated oral administration of carbon tetrachloride.

A quantitative histologic study was also made of the course of necrosis and repair in the liver following a single necrotizing dose. The percent of liver necrosis in relation to dose was determined.

While it was found that a correlation exists between the degree of liver necrosis and the incidence of hepatomas in relation to dose, the use of a graded series of necrotizing and nonnecrotizing doses indicated that repeated liver necrosis and its associated chronic regenerative state are probably not necessary for the induction of tumors with carbon tetrachloride.

Under the same experimental conditions, the minimum necrotizing and tumor-inducing doses for carbon tetrachloride were less than those for chloroform while the relative anesthetic activity of these two chemicals was of the reverse order.

During the course of chronic administration of carbon tetrachloride, the livers developed either relative or complete resistance or insensitivity (depending on the dose) to the necrotizing action of the agent; the hepatomas were completely insensitive or resistant. Following a single dose, the liver necrosis was uniformly centro-lobular. After chronic administration of the agent, the liver necrosis was around central veins, mid-zonal, or adjacent to portal veins and bile ducts.

There was a correlation between the dose of carbon tetrachloride chronically administered and the degree of lobular disorganization and amount of reticulum fibers deposited in the liver. Slight increase of reticulum fibers was observed after the chronic daily administration of sub-necrotizing doses of the agent.

[3] Unpublished data.

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QUANTITATIVE HISTOLOGIC ANALYSIS OF THE EFFECT OF X-RADIATION ON THE INTERSTITIAL TISSUE OF THE TESTES OF LAF_1 MICE

By ALLEN B. ESCHENBRENNER, *surgeon*, and ELIZA MILLER, *assistant biologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Observations on the effect of X-radiation on the testes are uniform in disclosing degeneration of spermatogenic elements as the most prominent feature. There is much difference of opinion, however, on the nature of the apparent change in quantity of interstitial tissue. It is obvious that there is a differential effect on these two chief components of the organ since the relative areas of the two in a histologic section are changed following irradiation. With decrease of cellularity and of the diameter of the tubules, there is an apparent increase of interstitial tissue. The question whether this apparent increase is real or relative does not appear to have been answered satisfactorily.

No attempt has been made to review the voluminous literature on this question, but in publications that have been surveyed differences of opinion and absence of quantitative data were found (*1*, *2*). The question is of considerable practical importance since radiation is used in investigations on interrelationships of various endocrine secretions (*2*, *3*). The present paper deals with the application of Chalkley's quantitative method for morphologic analysis of tissues to this problem. It was found that there is no absolute increase of interstitial tissue after whole-body irradiation of the mouse when doses sufficient to produce severe degeneration of spermatogenic elements are employed.

MATERIALS AND METHODS

LAF_1 hybrid male mice from colonies maintained at the National Cancer Institute were used. They were approximately 2 months of age at the beginning of treatment. The mice were irradiated for other purposes, hence the irradiation conditions were somewhat different in the two experiments reported herein, although not important for the present purposes. There were two groups of irradiated animals, one group that received a total dose of 300 r (air) over a period of 6 weeks and the other a total dose of 600 r (air) over a period of 11 weeks. Two groups of nonirradiated mice of the same ages were used as controls. Ten irradiated and ten nonirradiated mice were used in each experiment.

The whole-body irradiation was given under the following conditions: tube voltage, 170 kv. peak; tube current, 20.0 ma.; filter, 1.0 mm. copper and 1.06 mm. aluminum; and focus to middle of mouse, distance 105 cm. The dose of 300 r (air) was administered at the rate of 10 r in 1.82 minutes 5 times per week, and of 600 r (air) at the rate of 8.8 r in 1.65 minutes 6 times per week.

EXPERIMENTAL PROCEDURE AND RESULTS

Control and irradiated mice were killed by cervical dislocation 14 hours after the latter group had been irradiated. Both testes of each animal were carefully dissected free of the epididymis and fat and were weighed together on a torsion balance. They were fixed in Zenker-formol 4 to 5 hours, washed in running water 15 to 16 hours, and then placed in 80 percent ethyl alcohol. After several days, one testis of each animal was cut longitudinally and the other transversely. The halves were placed in 80 percent alcohol in a commercial tissue-changing device and dehydrated in progressive changes of alcohol, cleared in cedar oil, infiltrated with xylol-paraffin, and embedded in paraffin, m. p. 56° – 58°. The duration of exposure to each solution was the same for each group of tissues.

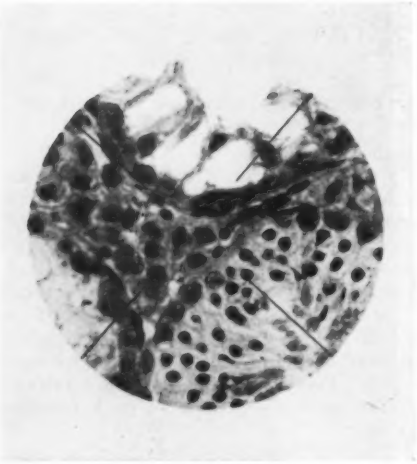

FIGURE 1.—Point pattern as seen in making measurements. Upper left pointer is on a connective-tissue-cell nucleus; the upper right on space; lower left pointer on the nucleus of an interstitial cell; the lower right one on the nucleus of one of the spermatogenic elements. The reading is 1 hit on interstitial tissue, 2 hits on other tissue, and 1 on space. From testis of a mouse exposed to 600 r. × 700.

In the embedding, a specially designed tray (*4*) was used, in which the temperature of the melted paraffin was controlled thermostatically, and the total time of exposure of the tissues to the hot paraffin was held within close limits. These precautions were taken to ensure that the shrinkage incident to the various procedures was as uniform as possible. All sections were cut 6μ in thickness. Mallory's aniline blue with acid fuchsin counterstain was used on all sections because of its clear differential staining of interstitial cell cytoplasm and other tissue elements.

Quantitative histologic analysis of the testes was carried out according to Chalkley's method (*5*). Sections were examined with a 4-mm., 0.95 N. A. apochromat objective (× 47.5) and × 15 compensating oculars. One ocular was fitted with four pointers made of fine silver wire. The small diameter of the pointers allowed rapid and easy identification of the tissue element that they touched. The arrangement of the pointers is shown in figure 1. One longitudinal section of one testis and one cross section of the other were each scanned with 250 microscope fields chosen at random in progressive series of paths, also chosen at random, in the two planes afforded by the mechanical stage of the microscope. At the margins of the sections, a field was used only if all pointers rested upon or within the limits of the capsule of the testis. Three types of ratios were determined: (1) Interstitial-cell cytoplasm or nucleus; (2) other tissue elements (spermatogenic elements, connective-tissue cells, or blood vessels, amorphous material, etc.); and (3) space due to shrinkage, regardless of its occurrence in relation to various tissue elements. One thousand random hits were made on each section, or a total of 2,000 on each pair of testes. A representative count of this type is shown in table 1. Duplicate counts were made in several instances by 2 different observers, with errors of less than 2 percent.

TABLE 1.—*Sample of Chalkley count on 1 longitudinal section of a testis of a mouse exposed to a total of 300 r* [1]

Tissue other than interstitial			Interstitial tissue			Space		
Hits	Accumulated total	Accumulated average	Hits	Accumulated total	Accumulated average	Hits	Accumulated total	Accumulated average
79	79	79.0	7	7	7.0	14	14	14.0
88	167	83.5	8	15	7.5	4	18	9.0
85	252	84.0	7	22	7.1	8	26	8.7
87	339	84.7	8	30	7.5	5	31	7.7
88	427	85.4	8	38	7.6	4	35	7.0
83	510	85.0	10	48	8.0	7	42	7.0
79	589	84.1	4	52	7.4	17	59	8.4
85	674	84.2	11	63	7.9	4	63	7.9
92	766	85.1	4	67	7.4	4	67	7.4
83	849	84.9	9	76	7.6	8	75	7.5

[1] The variation in individual groups of 100 hits and the relative uniformity of the accumulated averages as the total of 1,000 hits is approached, should be noted.

The percent of the area of each section comprising interstitial cells was

calculated by dividing the total number of hits on interstitial cells by the total number of hits on all tissue elements (disregarding hits on space). The weight of the pair of testes in milligrams was then multiplied by the percent of interstitial-tissue area for the sections of the testes, and a calculated total weight of interstitial tissue for the testes of each mouse was obtained. These values are given in table 2. Sections of a testis from a mouse of the 600-r group and of a normal mouse of the same age are shown in figure 2.

TABLE 2.—*Effect of 300-r and 600-r whole-body X-radiation on the testes of mice* [1]

Group	Body weight	Testes weight	Interstitial tissue	
	Grams	*Milligrams*	*Percent*	*Milligrams*
Controls for 300-r mice.	31	220	5.15	11.37
	32	210	5.81	13.13
	31	218	5.92	10.65
	31	204	4.68	9.91
	31	208	4.33	8.67
	34	221	4.38	9.65
	36	226	4.52	9.50
	33	180	4.32	9.41
	33	212	4.46	9.11
	30	200	5.19	10.79
Average	32±1.8	210±13.2	4.88± .60	10.22±1.09
300-r mice [1]	29	94	8.21	7.47
	27	90	8.50	9.69
	27	86	9.33	9.33
	28	72	9.39	8.26
	28	91	9.21	8.38
	29	114	7.84	7.37
	27	100	8.53	7.86
	27	88	8.68	7.42
	29	91	11.31	8.14
Average	28± .93	96±12.72	8.99±1.01	8.20± .98
Controls for 600-r mice.	39	237	5.57	13.22
	37	238	6.00	14.29
	32	224	4.12	9.24
	34	230	4.55	10.48
	31	236	5.10	12.04
	29	220	5.26	11.58
	33	240	5.52	13.24
	30	220	3.94	8.67
	31	200	6.17	12.35
	35	204	4.99	10.18
Average	33±3.16	225±14.14	5.12± .74	11.53±1.54
600-r mice	28	60	23.32	13.99
	35	68	19.17	13.03
	27	63	18.13	11.42
	30	56	21.91	12.26
	33	68	19.13	13.01
	26	54	12.53	6.76
	27	59	13.48	7.95
	33	64	19.17	12.26
	29	63	20.16	12.70
	29	51	26.97	13.75
Average	30±3.05	60.6±5.7	19.80±4.28	11.71±2.39

[1] 1 animal died before experiment was terminated.

DISCUSSION

In these studies several assumptions are made. It is assumed that a cross section or a longitudinal section of a testis is a reasonably representative picture of conditions prevailing in the testis as a whole. The ideal study would be to determine the relative areas of interstitial and other tissue in each of a complete set of serial sections. Obviously, this is out of the question for practical purposes; some limitation of detail must be exercised. It would not be too time-consuming to analyze 20 sections of a given pair of testes from a normal mouse and 20 sections of a pair from an irradiated mouse. It was considered, however, that the variation between different sections of a given testis would not be so large as the variation between the testes of different mice. For this reason, 2 sections from each of 10 irradiated and 10 control mice were studied for each of the 2 doses of irradiation. Since the main objective of the study was to compare the testes of irradiated and nonirradiated mice rather than to establish absolute values, and since it was decided to analyze a single section from each testis, a section through the maximum diameter of the organ was chosen.

The total weight of interstitial tissue per mouse was calculated by multiplying the fresh weight of each pair of testes by the percent of the total mass of the testes comprising this tissue as determined on fixed and dehydrated tissue histologically. It must therefore be assumed that the relative water content of irradiated and nonirradiated tissues was the same.

Testes that are exposed daily to the injurious effects of radiation and show extensive degeneration of spermatogenic elements might have a more active circulation than normal. This would be reflected in a slight discrepancy in the calculation of results. Blood vessels and their contents were included in the general category of tissue other than interstitial in our determinations. It would be hard to visualize that such a

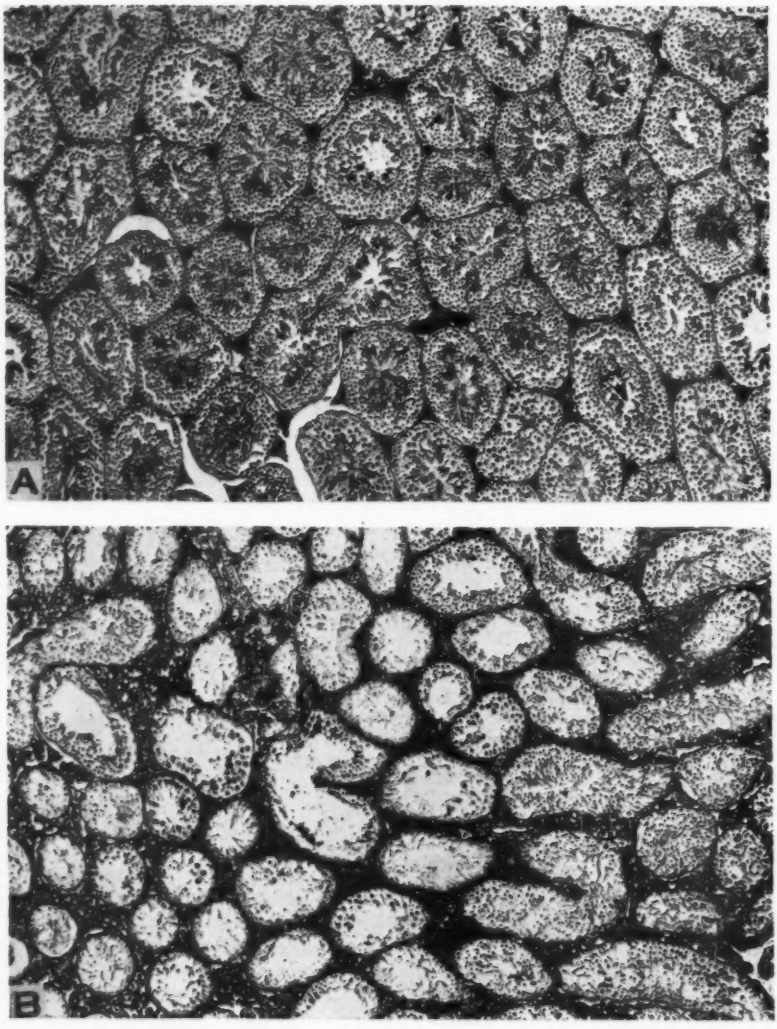

FIGURE 2.—Sections of testis of *A*, a normal mouse, and *B*, a mouse exposed to a total dose of 600r administered over a period of 2 months. Mallory aniline blue with acid fuchsin counterstain. Photographed with light green filter (interstitial tissue black). × 70.

discrepancy due to differences in circulation could nearly approach the magnitude of differences found in the relative percent of interstitial tissue in the control mice and those that had been exposed to a total dose of 600 r (5.12 ± 0.74 as compared with 19.80 ± 4.28). On the other hand, it would be hazardous to attempt to obtain any quantitative data on the relative circulation of the normal and experimental testes with the use of histologic sections prepared in the customary manner. Algire and Chalkley (*6*) in their studies on the vascular reactions of normal and malignant tissues in vivo point out that "the usual pathologic preparations give a completely erroneous idea of the vascular supply."

Chalkley's method is a valuable aid for studies such as these. Another method of approach would be that of making a large series of photographs or camera lucida drawings covering all of a section of the testis, followed by the measurement of relative areas of different tissue components by means of a planimeter. A complete or nearly complete set of such photographs or drawings would be necessary in view of the variation from field to field. To approach the fine differentiation of reticulum fibers and blood vessels associated with each small area of interstitial tissue under the high magnification possible with Chalkley's method, the number of photographs or drawings obtained with the alternative method would be so great and the procedure so time-consuming as to be prohibitive. In his original publication, Chalkley (*5*) showed a close correlation between the values obtained by using the two methods for determining nuclear-cytoplasmic ratios of liver cells.

A comparison of the calculated weight of interstitial tissue of the two groups of irradiated animals and their respective nonirradiated controls of the same age revealed a close correlation for the 600-r but not for the 300-r group. The latter had less interstitial tissue per animal than their controls. While ordinarily we consider male mice to be sexually mature at 2 months of age, when the irradiation was begun, the average testes weights for the control animals at 3 and 4 months of age were different, as were also the percent values for interstitial tissue determined histologically. We have quantitative data which show that the difference between 2 and 3 months of age is much greater.[1] We must assume that further sexual maturation takes place in the testes after 2 months and also after 3 months. The whole body irradiation might thus have retarded the rate of maturation, whether through local effects, general systemic effects, or both. In both series, the irradiated mice had a lower average body weight than had their respective controls of the same age.

This study emphasizes a fact that is generally understood but commonly over-looked: How much we depend upon relative rather than absolute values in viewing sections through the microscope. With the exception of the use of a comparison eyepiece fitted to two microscopes, in comparing sections we depend upon visual memory in passing from one slide to the next. We compare the size of a particular object with the size of the microscope field and with the size of other components of a section. The former comparison is complicated if we change from one magnification to another when a small portion of a section arouses our interest. With a lack of absolute values in such a comparison, anticipation of the fulfillment of our preconceived theories tends to have a distorting influence on our final conclusions. These hazards of microscopy can be eliminated by the use of Chalkley's method. A wider application of this method offers a partial solution to sorely needed quantitative methods in histology and pathology.

SUMMARY

The exposure of mice to doses of X-radiation sufficient to produce severe damage of spermatogenic elements re-

[1] Unpublished data.

sulted in an apparent increase of interstitial tissue. Chalkley's method for the quantitative morphologic analysis of tissues was used in a study of the testes of normal and irradiated mice. It was found that the apparent increase of interstitial tissue in the testes of irradiated mice is relative and not absolute.

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INCREASE IN INCIDENCE OF LUNG TUMORS IN STRAIN A MICE FOLLOWING LONG-CONTINUED IRRADIATION WITH GAMMA RAYS

By EGON LORENZ, *senior biophysicist*, WALTER E. HESTON, *senior geneticist*, MARGARET K. DERINGER, *research fellow*, and ALLEN B. ESCHENBRENNER, *surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Although the causal role of radiations in cancer of several organs and tissues is a well-established fact, the relation of radiation to pulmonary tumors of man and of experimental animals has not been proved. This problem is of considerable clinical importance as it is generally believed that the high incidence of pulmonary tumors of bronchiogenic origin in the miners of Schneeberg and Joachimsthal is caused by the radiation from radon inhaled over long periods. However, in a recent review by Lorenz (*1*) dealing with this problem, the author came to the conclusions that the opinion that radon is the sole cause of the lung cancer of the miners cannot be maintained, and that it is doubtful from the experimental material known at present whether or not radon can induce lung tumors in experimental animals. Long-term experiments, in which animals inhale radon, are difficult to carry out. However, since the primary reaction of all radiation is the same, namely, the formation of ion pairs in the tissues, a biologic end effect, such as tumor formation, if found with one radiation (α particles) will also be produced by other radiations (e. g., X- or γ radiation). It is difficult to ascertain whether or not the same amount of absorbed energy per gram of tissue will produce the same biologic effect as the ion-pair density along the path of an α particle is much greater than the ion density along the path of a photoelectron or recoil electron from X- or γ rays. Hence a minimum dose necessary to induce tumors with γ radiation may give only an upper limit for the necessary absorbed energy; and thus possibly smaller amounts of absorbed α-ray energy may produce the same effect.

The only available study in which the effects of X-rays and γ rays on pulmonary tumors in mice were noted was made by Furth and Furth (*2*). The authors subjected mice of different strains to massive doses of X-rays or continuous γ radiation for periods up to 2 years. However, differences between incidence of lung tumor in irradiated and control animals are not statistically significant.

In order to set up an experiment for the study of the effects of radiations (X-rays or γ rays) on pulmonary-tumor incidence, the following points should be considered. Heavy single or repeated doses of X-rays (1,000 to 10,000 r) will cause transient pneumonitis (Warren (*3*)), which makes the animals prone to infection. Long-continued exposures are preferred. Such exposures will make the experiments resemble more closely the conditions encountered in the continuous exposure of human beings. Furthermore, experimental animals should be chosen which have a high incidence of spontaneous lung tumors. Such animals are strain A mice, in which the incidence of spontaneous lung tumors has been studied (Bittner (*4*), and Heston (*5*)). In order to obtain statistically significant differences, comparatively large numbers of animals should be used. This makes it impracticable to submit lung tissues only to long-continued exposures. Total-body irradiation must be used, and the rate at which the dose is administered must be limited to such values that it does not considerably decrease the life span of the experimental animals.

MATERIALS AND METHODS

Sixty strain A mice (35 males and 25 females) were used as experimental animals, and the same number as controls. They were obtained from the National Cancer Institute stock in which Heston (5) studied the spontaneous incidence of pulmonary tumors. They were from 1 to 2 months old at the beginning of the experiment (May 10, 1944) and were divided among the experimental and control groups in such a way that the age distribution of the animals was the same in both groups. The mice were kept in wooden boxes, 8 to 10 animals in each. The boxes were made of pine board, 1 cm. thick, with inside dimensions of 28 x 18 x 15 cm. A small food hopper of galvanized iron was placed in the middle of each side board. The top of the boxes consisted of wire netting of 8-mm. mesh, nailed to a wooden frame. Drinking water was administered to the mice in wide-mouth bottles of 100-cc. capacity, closed by a rubber stopper, into which a glass tube approximately 8 mm. in diameter and 10 cm. long was inserted. The bottles were supported by the wire netting, with the drinking tubes approximately 5 cm. distant from the food hoppers along the midline of the cage. This arrangement was chosen to compel the mice to spend as much time as possible along the midline of the cage.

A detailed description of the irradiation arrangements will be given in a subsequent paper. For the purpose of this experiment, a brief outline is sufficient. The source of radiation was radium sulfate [1] contained in a capsule of platinum, 40 x 6.4 mm., 0.5 mm. thick. In equilibrium it has an external γ radiation equivalent to that of 992.5 mg. of radium element. The geometric center of each box of experimental animals was placed at approximately 80 cm. from the source. At this distance, the γ-ray intensity at the midpoint of the box was determined with Victoreen 0.25-r and 25-r [2] ionization chambers to be 1.1 rγ (air) per hour. Because of the varying distance of the different points inside the box from the source, the γ ray intensity varied from point to point, the maximum variations being approximately $\pm$ 10 percent. Since the mice were forced to feed and drink along the midline of the cages, the variations in total doses among the different experimental mice was probably somewhat smaller than $\pm$ 10 percent. The animals were irradiated for 8 hours daily. The experiment was terminated on February 21, 1945; at that time the surviving animals had been exposed for 287 days, and the total dose amounted to 2 500 rγ $\pm$ 10 percent.

RESULTS

Prior to the date on which the experiment was terminated, 17 irradiated animals and 5 controls had died from tumors or other causes or had been killed because of appearance of tumors. Histologic sections showed that 6 of these experimental mice (4 males and 2 females) had developed malignant lymphomas, 5 of which were of mediastinal origin. One male developed a salivary-gland tumor and one female a mammary carcinoma. Total doses given to these 6 animals ranged from 1,900 to 2,300 rγ. None of the 5 controls that died or were killed showed malignant lymphoma microscopically. Two had salivary-gland tumors.

When the surviving animals were killed, none showed malignant lymphoma grossly. Specimens of tissues other than lung tumors were taken only from a few animals, experimental and control, selected at random. Noteworthy histologic findings were absence of follicles in the ovaries and atrophy of spermatogenic elements of the testes.

To ascertain the incidence of lung tumors, the lungs of each animal were placed under a dissecting microscope, and the number of nodules appearing on the surface of the lungs of each ani-

[1] Loaned by the Canadian Radium and Uranium Corp. New York, N. Y.

[2] Calibrated by the Bureau of Standards with X-rays.

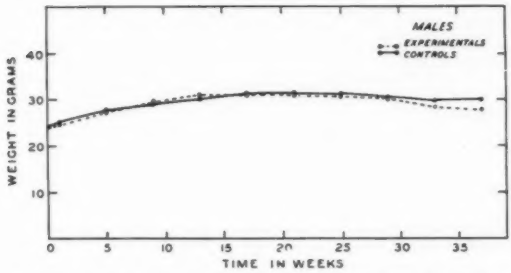

FIGURE 1.—Weight of experimental and control males.

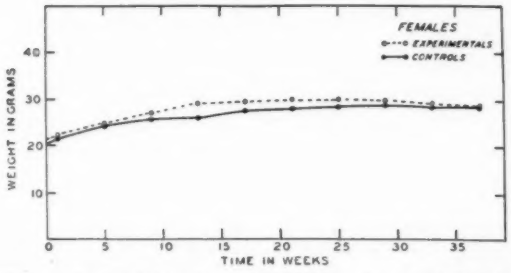

FIGURE 2.—Weight of experimental and control females.

mal was recorded. Each nodule was sectioned for histologic examination. The results, given in table 1, are based upon microscopic findings.

The increase of lung-tumor incidence in the experimental mice over that of the controls is statistically significant ($\chi^2=8.74$; $P \leqq 0.01$), as is the increase in animals with more than one lung tumor in comparison with animals with one lung tumor ($\chi^2=4.015$; $P \leqq 0.05$).

In the table, the animals are not separated according to sexes, as the distribution of males and females was approximately the same in the experimental and control animals. Furthermore, there is no significant sex difference in lung-tumor incidence according to Bittner (*4*). The lung-tumor incidence of 47 percent in the control animals is in agreement with the incidence of 53 percent given by Heston (*5*) for animals 12 months of age, and 42 percent given by Shimkin (*6*) for animals 10 months of age.

The weight of the experimental animals was apparently not affected by the irradiation. Figures 1 and 2 give the weight curves for the surviving males and females (experimental as well as

TABLE 1.—*Incidence of pulmonary tumors in experimental and control animals*

Group	Number at autopsy	Age	Mice showing given number of nodules per individual					Tumor incidence
			0	1	2	3	4	
		Months	*Number*	*Number*	*Number*	*Number*	*Number*	*Percent*
Experimental [1]	43	$10\frac{1}{2}$ to $11\frac{1}{2}$	10	17	7	6	3	76.7
Control [2]	55	$10\frac{1}{2}$ to $11\frac{1}{2}$	29	20	6	None	None	47.3

[1] 23 males and 20 females.

[2] 30 males and 25 females.

control animals). The animals were weighed once a week. The points plotted are the average of 4-week periods.

DISCUSSION

The carcinogenic action of γ radiation on lung tissue is difficult to understand, since lung tissue is only moderately radiosensitive. It is not likely that pneumonitis which follows heavy doses of radiation can play any important role with daily doses of 8.8 rγ per 8 hours. Histologic studies of normal lung tissue of these animals showed no sign of pneumonitis or any other change. The carcinogenic action of the radiation may be direct upon the lungs or it may be indirect through a systemic effect. Additional experiments are necessary to clarify this point. The experiments of Blum (*8*) give evidence against the formation of a permanent carcinogen by radiation.

It was pointed out earlier that different radiations differ only quantitatively in their biologic effect. Since γ rays increase the incidence of spontaneous pulmonary tumors in mice, α radiation emitted by radon and its decayed products should have the same effect. Hence the findings of Rajewsky et al. (*8*), who claim to have induced tumors of the bronchial epithelium of mice after inhalation of radon over a long period of time, are not supported by these experiments. There is no convincing evidence in the literature that spontaneous adenomas or adenocarcinoma of the mouse lung arise from bronchial epithelium (*9*).

What inferences can be drawn from these experiments as to the possibility that the incidence of lung tumors in man may be increased by long-continued irradiation? It is realized that any direct comparison between lungs of mice and those of human beings may not be justified because lung tumors in man are of bronchiogenic origin and those in mice are of alveolar origin, and the two may even have different etiologies. (1) It must be pointed out that, in comparison with the chemical carcinogens, radiation is of small potency as far as lung tissue is concerned. Intravenous injection of methylcholanthrene suspensions in amounts of 0.1 mg. of methylcholanthrene produces numerous lung tumors (*10*) although the carcinogen deposited in the lungs is rapidly removed (*11*). (2) It should be noted that in these experiments a strain of mice extremely susceptible to spontaneous pulmonary tumors was used. The incidence in strain A mice at 12 months of age is approximately 50 percent, and this increases to approximately 75 percent at 18 months of age. Compared with these data, the incidence of lung tumors in the human population is extremely low, smaller than 0.01 percent. Slye, Holmes, and Wells (*12*) make the following general statement:

> It seems that man exhibits lung tumors exceptionally infrequently, and that mice show an even more exceptional prevalence of tumors of this organ.

The dose of 2,500 rγ of whole-body irradiation used in these experiments exceeds by far the dose human beings receive, constantly exposed to a small daily dose, e. g., the tolerance dose of 0.1 r per day. In these experiments the total absorbed energy amounted to roughly 2×10^5 ergs per gram of lung tissue. The miners of Schneeberg or Joachimsthal were exposed daily to a concentration of radon of 3×10^{-9} curie per liter for 20 years on an average. The absorbed energy per gram of lung tissue amounted to approximately 1×10^3 ergs. It therefore seems that the high incidence of pulmonary tumors in the miners (approximately 50 percent of all deaths) cannot be attributed solely to the inhalation of radon.

The experiment, however, suggests that the carcinogenic action of radiation may also be weak on lung tissues of species other than the mouse and therefore not detectable in species with a low spontaneous incidence.

SUMMARY

Strain A mice of both sexes were exposed daily for 8 hours to radiation

(8.8 rγ). They were killed after 9½ months of daily exposure, having received a total dose of 2,500 r$\gamma \pm 10$ percent. The incidence of pulmonary tumors was approximately 50 percent higher in the experimental mice than in the controls. This result is statistically significant.

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SULFHYDRYL GROUPS IN THE CATHEPSIN OF RAT LYMPHOSARCOMA

By MARY E. MAVER, *senior biochemist*, and J. W. THOMPSON, *pharmacologist*, with the technical assistance of ANTOINETTE GRECO, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The functional role of the protein −SH group in the activity of many enzymes was the subject of critical reviews by Berson (*1*) and by Hellerman (*2,3*). Barron and Singer (*4–6*) recently found that many different enzymes which catalyze the carbohydrate, fat, and protein metabolism were proteins whose activities depended upon −SH groups. Earlier studies in this laboratory (*7–8*) on the effects of oxidation-reduction conditions upon the in vitro proteolysis of normal and malignant tissues showed that the −SH groups of the digests were correlated with the catheptic activity. The evidence presented here, that the endocellular proteinase or cathepsin of a neoplastic tissue is dependent upon the −SH groups of the protein-enzyme for hydrolysis of hemoglobin, is of particular interest in a study of the effect of oxidation-reduction conditions upon the proteolytic enzymes which probably function in the growth of normal and neoplastic tissues.

EXPERIMENTAL PROCEDURE

IDENTIFICATION OF SULFHYDRYL GROUPS

In establishing the essential role of the −SH groups in the protein-enzyme molecule, it was necessary to select a combination of reactions that would specifically identify the −SH radical. The so-called sulfhydryl reagents that have been used to identify this group may be divided into three general classes according to their mode of reaction:

(1) Oxidation of the −SH radical. In this group are found molecular oxygen with metal catalyst, iodine, iodosobenzoate, ferricyanide, and porphyrindine. The rate of this oxidizing reaction appears to be a characteristic of the −SH groups of the different protein-enzymes. The reversal of this reaction by reducing agents is limited to oxidations which have not proceeded beyond the disulfide stage and which, of course, have not attacked other parts of the protein-enzyme molecule.

(2) Reversible mercaptide formation. A group of derivatives of mercuribenzoic acid, prepared by Hellerman (*2*), has been found to be of exceptional service in the quantitative studies of several −SH enzymes. Sodium *p*-chloromercuribenzoate inhibited urease in high dilutions forming a reversible mercaptide.

Organic trivalent arsenicals form addition-compounds with −SH proteins which are reversible thioarsenites. Barron and Singer (*5–6*) used the derivatives of phenylarsenious acid, and they stated that the power of these arsenicals to combine with the −SH groups of some enzymes approached that of Hellerman's *p*-chloromercuribenzoate.

(3) Alkylation of −SH groups. Iodacetic acid and iodacetamide appear to react with the −SH group to form thioethers according to the following equation:

$$\mathrm{EnSH + ICH_2COOH \rightarrow EnSCH_2COOH + HI}$$

Intensive quantitative studies of the sulfhydryl groups in the proteins, especially egg albumin, by Greenstein (*9*) and Anson (*10*) showed that the different locations of the −SH groups in the protein molecule determine to what extent the sulfhydryl reagents will react in the case of each protein. Some proteins, such as egg albumin, give a positive nitroprusside test for −SH only after they have been denatured. On the other hand, native urease contains substituent −SH groups which continue to respond to the nitroprusside test after repeated recrystallizations of

the protein-enzyme (Sumner and Poland (*11*)). Furthermore, Hellerman, Chinard, and Deitz (*12*) found that all the −SH groups of urease were not essential for activity since iodacetamide or iodosobenzoate reacted with freely acting −SH groups without inhibiting the activity of the enzyme.

PREPARATION OF ENZYMES

The Murphy rat lymphosarcoma is particularly adapted to biochemical research because the rapidly growing tumor tissue can be readily separated from a small necrotic center. With slight variations, the procedures followed for the separation of the cathepsin from the lymphosarcoma were the same as those described earlier for normal and neoplastic tissues (*13*). The preparation was kept cold (8°–15°) throughout, and by avoiding the addition of acids or alkalies the pH range of the tissues and extracts was limited to 5.0–6.6. One kilo of pooled, frozen lymphosarcoma tissue was put through an electrically run meat grinder four times and then stirred mechanically with 5 l. of 0.1 M potassium chloride for 1 hour. The coarser tissue particles were removed by filtration through gauze, and the suspension was centrifuged in the refrigerated centrifuge. The pH of the supernatant fluid was 6.2. To the supernatant fluid 280 gm. of ammonium sulfate per liter was added to give 40-percent saturation. The precipitated globulin was first washed three times with cold 28 percent ammonium sulfate (the refrigerated centrifuge was used), and then was dialyzed against cold (10°) running tap water. Activity tests showed that the supernatant fluid in the dialyzing sacs contained most of the active cathepsin of the tissues. The protein fraction with catheptic activity could be precipitated by 40-percent saturation of the supernatant fluid with ammonium sulfate in most cases, although more ammonium sulfate was needed where the catheptic protein was in lower concentration. The precipitated catheptic fraction was dialyzed free from ammonium sulfate, and its solution was dried while frozen. The yields ranged from 400 to 600 mg. of dried preparation per kilo of frozen tumor tissue. These preparations showed approximately 17 times more catheptic activity per milligram nitrogen than did the original tumor tissues. Chemical analysis showed: Nitrogen, 15.32, 15.22 percent; sulfur, 1.65, 1.75 percent; phosphorus, trace; protein nitrogen, 11.98 percent; tyrosine color, 5.45 percent. Spectrographic analysis showed no more magnesium, manganese, iron, silicon, calcium, or copper than could be detected in once-distilled water.

DETERMINATION OF CATHEPTIC ACTIVITY

Three of the catheptic preparations made from lymphosarcoma tissues had the highest catheptic activity obtained as yet for rat-tumor tissue. In the present studies, two of these preparations were used, namely, lymphosarcoma cathepsin 2 and lymphosarcoma cathepsin 5.[1] The catheptic activities were determined by an adaptation of Anson's technique (*14*). In order to learn whether the possible peptidase activity and the proteinase activity were affected differently by the inhibitors or activators, both the tyrosine liberated and the protein hydrolyzed were determined on the same digests which had been incubated 90 minutes. This was considered ample time for the hydrolysis of the initial split products of the proteinase. Since the optimal hydrogen-ion concentrations for the hydrolysis of peptones and many synthetic peptides range from 4.2 to 5.5, the extent of peptide hydrolysis that would occur at pH 3.5 was not known.

In the pretreatment of the enzymes, 1 mg. of LSA2 or LSA5 was dissolved in buffered solutions, and graded amounts of inhibitor or activator were added in buffered solutions. The final volume of enzyme solution was made 1 ml. and held at room temperature for definite periods. To this 1 ml. of enzyme solution was added 5 ml. of

[1] Hereinafter referred to as LSA2 and LSA5, respectively.

warmed, freshly prepared 2.5 percent crystalline horse hemoglobin solution at pH 3.5. After 10 or 90 minutes' digestion at 39°, the unhydrolyzed protein in 1 ml. of the digest was precipitated with 10 ml. of 0.3 M trichloracetic acid. After standing 15 minutes at room temperature, the suspension was centrifuged and the supernatant fluid was filtered. The precipitates or in some cases aliquots of the filtrates were digested and analyzed for nitrogen by the micro-Kjeldahl technique of Koch and McMeekin (*15*). The tyrosine colors produced in aliquots of the filtrate by the addition of the Folin-Ciocalteau phenol reagent were read in a standardized Aminco electrophotometer. The tyrosine color could not be determined when glutathione or trivalent arsenic compounds were in the digests since these reagents reduce the phenol reagent.

INHIBITORS OF CATHEPTIC ACTIVITY

The *p*-chloromercuribenzoic acid used was prepared by Dr. Leslie Hellerman,[2] of the Johns Hopkins University School of Medicine. Commercial *o*-iodosobenzoic acid was purified according to the method of Hellerman, Chinard, and Ramsdell (*16*). The iodacetamide was prepared in this laboratory from chloracetamide and sodium iodide according to the procedure of Braun (*17*). The trivalent organic arsenical, 3-amino-4-hydroxyphenyldichlorarsine hydrochloride was obtained from the Division of Biologics Control, National Institute of Health.

The concentrations of inhibitors and activators are given in micromoles ($M \times 10^{-6}$) per milligram of enzyme in 1 ml. buffer. The percent of inhibition was calculated by using the activity at pH 3.5 or pH 7.0 as 100 percent.

REACTIVATION EXPERIMENTS

After the inhibitors had been in contact with the enzymes in buffered solutions for 20 or 60 minutes at room temperature, different quantities of glutathione in neutral solution were allowed to remain in contact with the enzymes plus inhibitor for 10 minutes before addition of the substrate. The effect of the same amounts of glutathione on the enzymes without inhibitor was determined.

RESULTS

TITRATION OF SULFHYDRYL GROUPS IN LYMPHOSARCOMA CATHEPSIN

The total sulfhydryl groups in the lymphosarcoma-cathepsin preparations were titrated with sodium iodosobenzoate after denaturation with guanidine hydrochloride according to the method described by Hellerman, Chinard, and Deitz (*12*) for urease. To a solution of 10 mg. of the dried cathepsin preparation in 0.5 ml. M/15 phosphate buffer at pH 7.0 were added 1.5 ml. water, 0.5 ml. M buffer at pH 7.0, and 1 gm. neutral guanidine hydrochloride. After 30 minutes' standing under nitrogen at room temperature, 3 ml. of 0.00204N sodium iodosobenzoate was added. After 2 minutes, 0.5 gm. potassium iodide dissolved in 3 ml. N hydrochloric acid was added, and the iodine was titrated with 0.00208N sodium thiosulfate: 2.42 and 2.45 ml. thiosulfate were required. These titrations indicated the presence of 1.09 and 1.03 micromoles of −SH in 10 mg. of enzyme preparation.

With the use of an outside nitroprusside-test end point, the −SH groups of 10 mg. lymphosarcoma-cathepsin preparation, which was denatured with guanidine hydrochloride, were found to combine with 0.93 ml. of 0.001 M *p*-chloromercuribenzoate. This indicated the presence of 0.93 micromoles of −SH per 10 mg. of enzyme preparation.

When freshly prepared, the LSA2 and LSA5 gave faintly positive nitroprusside reactions in the undenatured state, but these reactions were negative within 1 month after preparation.

INHIBITION OF CATHEPTIC ACTIVITY BY ACID AND ALKALI

The activities of the lymphosarcoma cathepsins were inhibited to some extent by exposure to acid, neutral, and

[2] We are indebted to Dr. Hellerman for generously furnishing this compound.

alkaline buffers (tables 1 and 2). This was a complicating factor since most inhibitors required a neutral or alkaline medium for reaction. In some cases, when the exposure to alkali had not been prolonged, these inhibited enzymes could be partially or fully reactivitated by the addition of buffered solutions of glutathione. This inhibiting effect might have been the result of the oxidation of sensitive −SH groups on the enzyme-protein molecule. On standing in alkali, further disruption of the protein might have occurred. The experiments of Schäffner and Truelle (*18*) demonstrated the effects of acid and of alkali upon the specific activities of beef-liver-cathepsin preparations.

TABLE 1.—*Inhibition of catheptic hydrolysis of hemoglobin by iodine, iodosobenzoate, and iodacetamide*

Enzyme	Pretreatment of enzyme			Hydrolysis					Reactivation		
	pH	Length of exposure	Concentration of inhibitor	Digestion period	Tyrosine liberated		Protein nitrogen hydrolyzed		Concentration glutathione	Protein nitrogen hydrolyzed	
					Amount	Inhibition	Amount	Inhibition		Amount	Percent
		Minutes	*Micromoles*	*Minutes*	*Mg.*	*Percent*	*Mg.*	*Percent*	*Micromoles*	*Mg.*	
IODINE											
LSA 5 (1 mg.)	3.5	0	0	10	0.687	0	1.40	0			
	3.5	60		10	.557	18.9	1.104	21	1	1.39	Complete.
	3.5	60	.25	10	.443	35.5	.768	45.1	1	1.39	Do.
	3.5	60	.50	10	.264	61.5	.738	47	1	1.38	Do.
	7.0	0	0	10	.399	41.8	.840	40.0	3	1.20	82.1
	7.0	60	.125	10	.228	66.8	.720	48.5	3	.864	21.1
	7.0	60	.25	10	.163	76.2	.610	56.4	3	.720	13.1
LSA 2 (1 mg.)	3.5	0	0	90	1.386	0	2.58	0			
	3.5	60	0	90	1.067	23.0	1.86	27.9			
	3.5	60	2.0	90	.733	47.0	1.08	58.1			
	7.5	60	0	90	.957	30.9	1.840	28.6			
	7.5	60	.20	90	.739	46.7	.924	64.1			
	7.5	60	.50	90	0	100	.660	76.7			
	7.5	60	1.00	90	0	100	0	100			
IODOSOBENZOATE											
LSA 5 (1 mg.)	3.5	0		90	1.23		3.08				
	7.0	20	0	90	.965	21.5	2.16	26.6	3.2	2.70	
	7.0	20	1	90	.882	28.29	1.77	42.5	3.2	2.74	74.0
	7.0	20	2	90	.844	31.3	1.82	40.9	3.2	1.82	0
	7.0	20	8	90	.561	54.3	1.32	57.1			
	7.0	120	0	90	.835	32.0	1.27	58.7	20	1.82	
	7.0	120	.5	90	.723	41.2	1.30	59.8	20	1.54	13.4
	7.0	120	2	90	.704	42.0	1.38	55.1	20	1.42	2.3
	7.0	120	4	90	.633	48.5	1.34	56.5	20	1.34	0
	7.0	120	8	90	.479	61.05	.828	73.0			
	7.0	120	10	90	.389	68.3	.900	70.0			
LSA 2 (2 mg.)	3.5	60	0	10	1.37	0					
	3.5	60	5	10	1.110	19.2					
	7.0	60	0	10	.953	30.7					
	7.0	60	5	10	.916	33.2					
IODACETAMIDE											
LSA 5 (2 mg.)	3.5	0	0	10	1.122	0					
	7.0	60	0	10	.949	15.4					
	7.0	60	5	10	.853	20.4					
LSA 5 (1 mg.)	3.5	0	0	90	1.23	0	3.08	0			
	7.0	60	0	90	1.15	6.5	2.53	17.8			
	7.0	60	5	90	1.04	15.4	2.24	27.2			
	7.0	60	10	90	.910	26.0	1.94	37.1			
	7.0	60	20	90	.851	30.8	1.74	43.5			

TABLE 2.—*Inhibition of catheptic hydrolysis of hemoglobin by p-chloromercuribenzoate and 3-amino-4-hydroxy phenyldichlorarsine hydrochloride*

P-CHLOROMERCURIBENZOATE

	Pretreatment of enzyme			Hydrolysis							
					Tyrosine liberated		Protein nitrogen hydrolyzed		Reactivation		
	pH	Length of exposure	Concentration of inhibitor	Digestion period	Amount	Inhibition	Amount	Inhibition	Concentration of glutathione	Protein nitrogen hydrolyzed	
										Amount	Percent
		Minutes	*Micromoles*	*Minutes*	*Mg.*	*Percent*	*Mg.*	*Percent*	*Micromoles*	*Mg.*	
LSA 5 (1 mg.)	5.0	20	0	90	1.43	0	2.45	0	20	2.49	
	7.0	20	0	90	1.29	9.8	1.93	21.2	20	2.80	
	7.0	20	.05	90	.91	36.5	1.53	37.9	20	2.56	Complete
	7.0	20	.05	90	.91	36.5	1.53	37.9	10	1.75	23.8
	7.0	20	.05	90	.91	36.5	1.53	37.9	5	1.48	0
LSA 2 (1 mg.)	7.0	20	0	90	1.115	0	3.02	0	20	3.38	
	7.0	20	.05	90	.919	17.5	2.05	32.1	20	2.80	77.3
	7.0	20	.10	90	.660	40.8	1.94	35.7	20	2.34	37.0
	7.0	20	.20	90	.372	66.6	1.02	66.3			
LSA 5 (2 mg.)	3.5	60	0	10	1.327	0					
	7.5	60	0	10	1.18	10.7					
	7.5	60	.0125	10	1.10	17.1					
	7.5	60	.0250	10	.930	29.9					
	7.5	60	.0500	10	.846	36.2					
	7.5	60	.096	10	.584	55.9					
	7.5	60	.200	10	.302	77.2					
LSA 2 (1 mg.)	7.5	120	0	90	.605	54.4					
	7.5	120	.1	90	.121	91.0					
	7.5	120	.20	90	0	100					

3-AMINO-4-HYDROXYPHENYLDICHLORARSINE HYDROCHLORIDE

LSA 5 (1 mg.)	7.5	20	0	90			2.70	12.3	20	2.90	
	7.0	20	1	90			2.30	25.3	20	2.77	Complete.
	7.0	20	5	90			2.15	30.2	20	2.83	Do.
	7.0	20	10	90			2.16	29.8	20	2.83	Do.

INHIBITION OF CATHEPTIC ACTIVITY BY IODINE AND SODIUM IODOSOBENZOATE

Iodine, which is known to be a powerful oxidizing agent, inhibited the catheptic activity in high dilutions when in neutral or alkaline mediums (table 1). In the acid solution, pH 3.5, much more iodine was required for the inhibition of the activity and oxidation of the $-SH$ groups. Reactivation with excess glutathione was practically complete in the case of the enzymes oxidized in acid medium but was very slight in the cathepsins oxidized at pH 7.0 where the sulfhydryl groups may have been oxidized beyond the disulfide stage or where other groups on the protein-enzyme may have been attacked.

Increasing inhibitory effects were also found in the treatment of the enzymes with increasing quantities of the oxidizing agent, sodium iodosobenzoate (table 1).

There was no evidence that the oxidizing inhibitors had affected the hydrolysis, as measured by the tyrosine liberated, differently from that measured by the hydrolysis of the protein nitrogen. The fact that the percentage inhibitions measured by both methods were in fairly close agreement might indicate that the same $-SH$ groups were essential for the peptidase and proteinase hydrolysis of hemoglobin at pH 3.5. It was interesting that the hydrolysis in the first 10 minutes was equivalent to approximately one-half the hydrolysis in 90 minutes.

INHIBITING EFFECT OF IODACETAMIDE

Iodacetamide was not effective as an inhibitor in dilute solution (table 1). When 20 micromoles of iodacetamide was used, only 43-percent inhibition of the catheptic activity was obtained. With these large quantities of inhibitors, groups other than the −SH groups might have been affected. Anson (*19*) reported that the proteinase activity of a preparation of liver cathepsin was not inhibited by 0.01 iodacetic acid at pH 2.7, when casein and globin were used as substrates. Later Anson (*20*) found that the activity of an alkaline solution of beef-spleen cathepsin was not inactivated by iodacetamide and concluded that cathepsin is "not a proteinase of the papain type," that is, a sulfhydryl enzyme. More recent evidence, by Hellerman and co-workers (*12*) on urease and by Singer and Barron (*21*) on adenosinetriphosphatase, has proved that the alkylating reagents, iodacetic acid and iodacetamide, alone, cannot be relied upon to determine the essential role of the −SH groups of an enzyme.

INHIBITION OF CATHEPTIC ACTIVITY BY *p*-CHLOROMERCURIBENZOATE

Extensive inhibition of catheptic activity was obtained with relatively dilute concentrations of *p*-chloromercuribenzoate (table 2). The enzymatic titration of the −SH groups showed that 0.1 micromole of *p*-chloromercuribenzoate in pH 7.5 buffer for 2 hours reduced the activity of 1 mg. LSA5 91 percent. This agreed very well with the stoichiometric titration with sodium iodosobenzoate, which showed that 10 mg. LSA5 contained 1 micromole −SH. This correlation of the enzymatic and stoichiometric titrations was based on the assumption that the mercury-sulfur-enzyme complex was not affected by the alkaline buffer.

The reversibility of the enzyme mercaptide reaction was readily demonstrated when excess glutathione was added within 20 minutes after the inhibitor.

The maximum inhibition obtained with 20 micromoles of 3-amino-4-hydroxyphenyldichlorarsine was 30 percent, and this inhibition could be completely reversed by adding excess glutathione. The relatively large quantity of arsenical required for this limited inhibition indicated that the essential −SH groups of the lymphosarcoma cathepsin did not react extensively with this sulfhydryl agent.

ACTIVATION OF CATHEPTIC ACTIVITY WITH SODIUM CYANIDE

The proteinase activities of the lymphosarcoma cathepsins as measured by the decrease in precipitable protein of hemoglobin were found to be activated by cyanide, while the tyrosine liberated in the same digests was only slightly increased (table 3). The optimal concentration for activation of the proteinase activity was 10 micromoles per milligram enzyme per milliliter, corresponding to 0.01 M cyanide. The activation of proteinase activity was enhanced by anaerobic conditions. The activation can probably best be explained as due to the reduction of disulfide bonds in the enzyme, since after treatment with cyanide the nitroprusside test for −SH groups became positive. The absence of a corresponding increase in the amount of tyrosine color liberated may be similar to the inhibiting effect of cyanide on the peptidase activity of papain extracts, which was reported by Ambros and Harteneck (*22*).

The activation of enzymes by the reducing action of cyanide has not been interpreted as clear-cut evidence of the presence of essential −SH groups (Hellerman (*2*)). Bersin (*1*) demonstrated that the action of cyanide was not a simple reduction, when he found that the treatment of a dithiol compound with cyanide yielded one −SH group and a thiocyanate compound. The lymphosarcoma-cathepsin preparations were essentially free of heavy metal contaminants, and therefore the cyanide would not activate the enzyme by removing a metal inhibitor from its mercaptide formation. But it is possible that other groups than the −SH

TABLE 3.—*Activation of lymphosarcoma cathepsin 2 by cyanide*

Pretreatment of enzyme			Hydrolysis of hemoglobin							
				Tyrosine liberated				Protein nitrogen hydrolyzed			
				Aerobic		Anaerobic		Aerobic		Anaerobic	
pH	Length of exposure	Concentration of cyanide	Digestion period	Amount	Increase	Amount	Increase	Amount	Increase	Amount	Increase
	Minutes	*Micromoles*	*Minutes*	*Mg.*	*Percent*	*Mg.*	*Percent*	*Mg.*	*Percent*	*Mg.*	*Percent*
3.5	60	0	90	1.43		1.40		3.42		3.42	
3.5	60	5	90	1.54	7.7	1.51	5.5	3.82	11	3.98	16.37
3.5	60	10	90	1.51	5.5	1.55	8.3	4.80	40	5.76	68.4
3.5	60	20	90	1.67	16.7	1.67	16.7	4.68	36.8	5.24	53.2
3.5	60	40	90	1.43	0	1.40	0	4.35	27.1	4.50	31.5

groups might be acted upon by the cyanide.

DISCUSSION OF RESULTS

The essential role of the substituent −SH groups of the cathepsin of rat lymphosarcoma in the hydrolysis of hemoglobin has been best demonstrated by the oxidation reactions with iodine and sodium iodosobenzoate and by the inactivation with *p*-chloromercuribenzoate which was reversed by the addition of glutathione. At present, no other protein radical is known which enters into these two reactions, oxidation and mercaptide formation. The proteinase activity studied here was the result of the splitting of many different peptide bonds in the hemoglobin. It will be of interest to investigate the role of the −SH groups in the peptidase activities of cathepsins on those synthetic peptides which have been shown by Fruton, Irving, and Bergman (*23*) and others (*18*, *24*) to be activated by cysteine, glutathione, and ascorbic acid. The divergent results in activating and inactivating experiments with the cathepsins have probably been caused by the different methods of preparation, which have not been designed to preserve the −SH groups of the protein-enzyme.

While in these experiments only one cathepsin was studied, other less extensive inhibitory tests have suggested that the cathepsins of many normal and neoplastic tissues may be sulfhydryl enzymes. Since the cathepsins function in the lysis and presumably in the synthesis of tissue proteins, the physiologic conditions that influence the −SH groups of cathepsins would control the growth of cells. It might be said that the most effective control of the activity of cathepsins and other −SH enzymes is provided by the oxidation-reduction potentials in the cell. These in turn are regulated by the pH, substrate hydrogen, oxygen, various enzyme catalysts (such as flavo- and pyridin-ademine nucleotides), glutathione, and ascorbic acid.

SUMMARY

Purified preparations of the cathepsin of the Murphy rat lymphosarcoma were tested with several sulfhydryl reagents and were found to contain −SH groups which were essential for activity. These groups were oxidized, and the enzymes were inhibited with iodine and sodium iodosobenzoate. The cathepsin was also reversibly inactivated by *p*-chloromercuribenzoic acid. The quantity of *p*-chloromercuribenzoic acid needed for complete inactivation of the cathepsin was closely correlated with the total quantity of −SH groups found by chemical titration of the cathepsin with sodium iodosobenzoate after denaturation with guanidine hydrochloride. The −SH groups essential for catheptic activity did not combine extensively with 3-amino-4-hydroxyphenyldichlorarsine hydrochloride or readily with the alkylating reagent, iodacetamide. The cathepsin was activated with sodium cyanide.

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EFFECT OF THE LEVEL OF DIETARY PROTEIN ON VASCULAR REPAIR IN WOUNDS

By HAROLD W. CHALKEY, *senior physiologist*, GLENN H. ALGIRE, *senior assistant surgeon*, and HAROLD P. MORRIS, *senior nutrition chemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Several authors have studied the effect that the level of the protein intake has on wound healing. Clark (*1*) reported evidence that maintenance on a high protein intake increases the rate of closure of skin wounds in the dog. Harvey and Howes (*2*) asserted that maintenance on a high protein diet increases the rate of wound healing as measured by the increase in tensile strength of the union. This contention is also supported by the evidence of Thompson, Ravdin, and Frank (*3*) that a reduction of only 15 percent in plasma protein may lead to a moderate delay in wound healing, whereas a reduction of 25 percent is sufficient to cause very serious delay or complete failure.

For the movement and growth of the epithelial tissue in the closure of wounds, a sound bed of granulation tissue is essential; and it is quite obvious that the growth of all tissues concerned is dependent on their nutritional supply, which in the higher organisms is furnished through the blood stream. It therefore follows that a study of the repair of the blood supply following trauma, if it could be made quantitative, might be expected to serve as a criterion of the basic process in tissue repair.

While the importance of such a criterion is realized, and studies along this line have been attempted, they have failed to give at one and the same time a dynamic picture, both morphologic and quantitative, of the progress of repair in the traumatized vascular bed. To illustrate, Hughes and Dunn (*4*) presented a method for the quantitative study of vascular regeneration in experimental wounds, using injection methods, together with the ordinary pathologic techniques. This method, while a step forward, is limited on the side of morphology to static pictures of a series of discontinuous stages obtained from different animals killed at various intervals and cannot give a quantitative demonstration of the in vivo fluctuations of the vascular bed during the repair process. It can only roughly delineate the rate of progress of the vascular network into the damaged area and would be quite inadequate for dealing with a condition in which the vascular tissue is damaged but not eliminated. Nor can it appraise quantitatively a difference between the vascular beds of the undisturbed and the newly repaired areas of tissue once the vessels in these are conjoined.

The present authors had previously developed methods which it seemed would enable them to follow visually the processes of repair in vivo, and a method which made it possible to ascertain the ratios of tissues in any area and to compare them with the like ratios in adjacent areas.

The problem as the authors saw it was, therefore, by use of these methods to ascertain in vivo the effect of the protein level in the diet on vascular repair.

MATERIALS AND METHODS

The animals used were adult male strain C mice 4 to 6 months of age weighing approximately 25 gm. each. All mice were caged separately, and a record was kept for each mouse, of food intake and fluctuations in weight for at least 3 weeks prior to as well as during the period of experiment. Before wounding, weights were recorded twice a week; but during the period of healing all animals were weighed before and after each observation.

The diets used are given in table 1. They are designated low protein, nor-

mal,[1] and high protein and are so referred to henceforth. Lactalbumin was used as a source of protein because of its high biologic value in regeneration of serum protein. The lowest level (7 percent) of lactalbumin was selected as a minimum as it will barely maintain body weight. The 14-percent level was considered normal. Sixty percent was arbitrarily set for the high-level diet. No data were available to determine the maximum percentage of protein upon which the mice would thrive. However, animals fed at this level for 3 months or more did not appear to thrive so well as those fed this high-protein diet for only a few weeks.

The general plan of the experiment was as follows: The animal selected for experiment was anesthetized, and then a transparent chamber was inserted in a flap of skin. The operation was substantially that described by Algire (*5*). The resultant chamber is shown in figure 1.

At the time of operation and just prior to the insertion of the chamber, a wound was made in the area that subsequently lay within the chamber, as follows: In the exposed subdermal tissue,[2] an area was selected that was free from large vessels. An impression was made in this area by light pressure with a hollow circular punch 3 mm. in diameter. Then under a dissecting microscope, the disk of tissue thus outlined was carefully excised by use of iridectomy knives and jewelers' forceps. The incision was made so that the disk of tissue removed consisted only of superficial fascia, underlying muscle layer,

TABLE 1.—*Composition of diets*

Constituents	Diet		
	Low protein	Normal	High protein
	Percent	*Percent*	*Percent*
Lactalbumin [1]	7.0	14.0	60.0
Cerelose (corn dextrose)	61.0	54.0	8.0
Lard	10.0	10.0	10.0
Corn oil	10.0	10.0	10.0
Wheat-germ oil	.5	.5	.5
Cod-liver oil	2.0	2.0	2.0
Salt mixture (185a) [2]	4.0	4.0	4.0
Choline hydrochloride	.5	.5	.5
Ryzamin B	4.0	4.0	4.0
Dextrose containing 1 mg. riboflavin	1.0	1.0	1.0

[1] Borden technical grade.
[2] See Morris and Robertson (*6*).

[1] The term "normal" as used herein refers to the diet containing 14 percent protein.

[2] The order of the tissues is of course reversed from that seen in a wound made by passing through the epidermis from without, i. e., from upper to lower layer, superficial fascia, muscle, connective and adipose tissues containing hair follicles, and epithelium.

FIGURE 1.—Transparent chamber in place in a dorsal skin flap in the mouse. Arrow indicates the site of the wound.

and a portion of the subcutaneous adipose and connective tissues, thus providing an aseptic wound still protected by the epidermis, and one in which the vascular structures were fully exposed for observation and measurement. Great care was exercised not to interfere with the epidermis or hair follicles.

Immediately after operation (except as noted hereafter), measurements were made of the relative level of vascular tissue (in terms of volume percentage of the total tissue observed) both in the wound and in the surrounding connective tissue. The method used was that described by Chalkley (7).[3]

Such measurements were made daily thereafter [4] (except Sundays) until the termination of the experiment.

A considerable number of measurements were made of normal tissue in such chambers before regular experimentation was started in order to test the degree of reproducibility of the measurements. The results, given in figure 2, show excellent consistency. A statistical study was made of some data accumulated during the progress of the research, which showed that the average standard deviation for single values is approximately 10 percent of the mean for the range of values from 10 to 60 percent. In all experiments, at least 125 observations (giving 500 possible hits) were made to establish a single recorded value. The time necessary was about 5 minutes.

Photographs of the chamber as a whole at 10 × magnification and of the wound area at 35 × were made at fairly regular intervals. In making them, care was exercised to include the stages of the repair curve where experience indicated that marked changes in the behavior of the tissues were to be expected (i. e., the end of the latent period and the point of stabilization).

Plots were made on which from day to day the changes in the vascular levels in the individual wounds and the surrounding tissues were recorded. In these wounds there were noted the time of appearance of new tips (capillary sprouts) and the onset and complete gelation of plasma in the wound area.[5]

EXPERIMENTAL PROCEDURE

The experiment was designed to answer the following questions: (1) Do

[3] In this method, a microscope fitted with an eyepiece arranged to show a fixed pattern of points in the focal plane is used. Repeated presentation of this pattern to the tissue is made, and the visual contacts of the points with the tissue to be measured are recorded. The ratio between the number of contacts with the tissue measured and the total chances of contact in the volume covered is directly proportional to the volume ratios between the tissue and that total volume. The absolute volume values are not needed.

[4] The times of operation were so selected that no known crucial stage in the repair process would be expected to occur on a Sunday.

[5] For a detailed morphologic description of the repair process as observed in the chamber, see Algire and Chalkley (8).

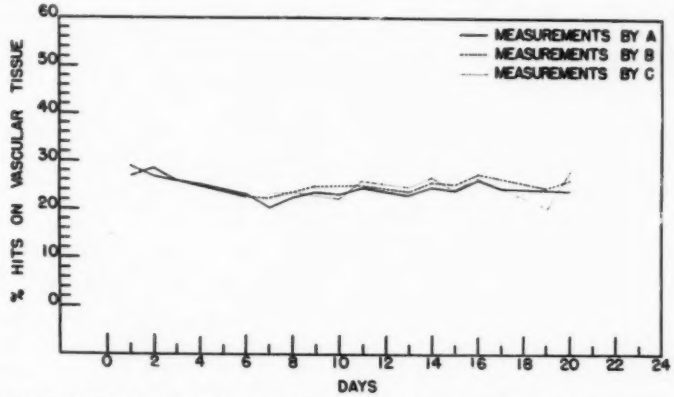

FIGURE 2.—*Reproducibility of the measurements of the vascular bed in connective tissue.*

wounds of the type used restore their vascular bed more rapidly in animals maintained on a diet containing a high-protein content than similar wounds in animals on a diet containing a low percentage of protein, or than those in animals on a diet containing an intermediate (normal) percentage of protein? (2) If positive results are obtained, can they be obtained also with large increases in the protein intake immediately subsequent to infliction of the wound?

Preliminary experiments were carried out to obtain criteria for determining whether a particular wound should be included in the composite data. It was found that infection, excessive damage to the tissues during operation, post-operative hemorrhage, accidental inclusion of air bubbles, and intercurrent diseases were all factors that tended to delay vascular repair and therefore interfered with proper evaluation of the factors under consideration. The animals in which any of these disturbing conditions appeared were hence excluded from consideration.

The first experiment includes the results obtained with six mice fed the normal diet.

In figure 3, the average values obtained for the measurements of functional (i. e., undamaged) and also of total vascular tissue in the wound throughout the experimental period, together with values from the vascular level in the surrounding uninjured tissues are shown as plotted against the time in days after the operation at zero time. In the graphs depicting the vascular changes in wounds, the ordinate values are shown as percentages of the average value of all the measurements made on connective tissue throughout the experiments. This method was adopted to secure a basis for comparison of all experiments. (In the graph showing reproducibility, however, the ordinates are the actual values in hits on connective tissue, i. e., in volume percentage.) Also noted on this figure is the day when on an average new vascular tips were first definitely noted in the wound area. The intersection of the curve for the regenerating bed and that for the normal surrounding tissue in this as in all other experiments is taken to indicate the time of completion of vascular repair. In this instance it was 9.8 days after operation. New tips were first seen on the sixth day.

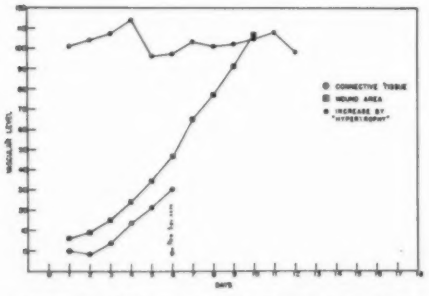

FIGURE 3.—Changes in the vascular bed during wound healing in mice fed a 14-percent protein diet prior to and throughout the experiments (averages for 6 mice).

In this and all other experiments here reported, a definite increase in active vascular bed was evident and measurable before definite new tips were seen. Close observation has convinced us that part of this is due to reactivation of nonfunctional tissue. However, neither in the wounds shown in figure 3 nor in those shown subsequently, is the reactivation (decline of nonfunctional tissue) sufficient to account for the whole of the rise in vascular bed that occurred before new tips were formed. The course of this rise in vascular bed prior to the formation of new tips is shown in figures 3–7 (collectively in fig. 9). Calculations were made of this excess vascular bed as follows: Taking the total percentages of first functional and second nonfunctional vascular tissue as of the first day as a base, we find (1) that the increase in this percentage of functional tissue at any given later time is the percentage at that time less the original (first day) percentage of functional tissue, and (2) that the percentage of recovered nonfunctional tissue, at any given time, is the percentage of nonfunctional tissue of the first day less

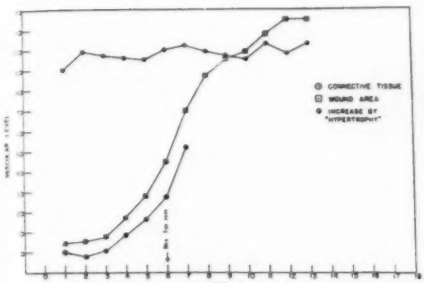

FIGURE 4.—Changes in the vascular bed during wound healing in mice fed a high-protein diet for a short time (15 days) prior to experiment and maintained on that diet throughout the experiment (averages for 6 mice).

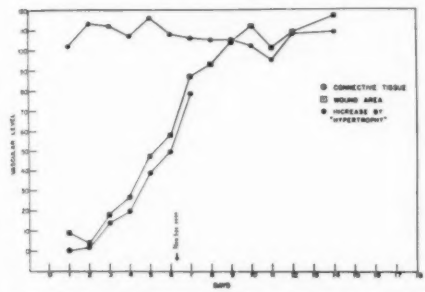

FIGURE 5.—Changes in the vascular bed during wound healing in mice fed the normal diet up to the time of wounding and then transferred to and maintained on the high-protein diet for the remainder of the experimental period (averages for 6 mice).

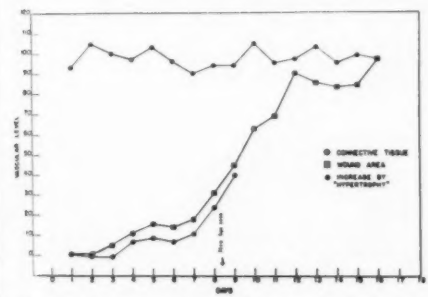

FIGURE 6.—Changes in the vascular bed during wound healing in mice fed the high-protein diet for an average of 45 days prior to wounding and maintained on that diet throughout the experiment (averages for 5 mice).

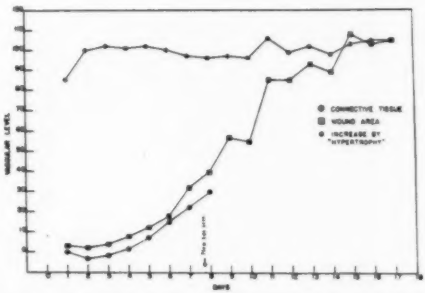

FIGURE 7.—Changes in the vascular bed during wound healing in mice fed the low-protein diet prior to and throughout the experiment (averages for 6 mice).

the nonfunctional tissue at the given time. A difference between (1) and (2) where (1) is the larger must mean that more tissue is present than can be accounted for by the recovery from damage. For the purposes of this report, we have designated this excess "increase by hypertrophy" when unaccompanied by the formation of new tips, i. e., when it occurred before the appearance of new tips. This may be expressed as

$$(a - a_o) - (b_o - b)$$

where

a_o is functional tissue on the first day;
b_o is nonfunctional tissue on the first day;
a is functional tissue on any given day;
b is nonfunctional tissue on any given day.

Further consideration of this point is given in the discussion.

In the second experiment, six mice were used. They were fed the high-protein diet 11 to 19 days (average 15.5 days) before the operation. The results are shown in figure 4. The intersection of wound and normal curves occurred on the average 9.4 days after the operation, and new tips appeared on the sixth day.

The third experiment includes the results obtained with six mice fed the normal diet until the day of operation and changed subsequent to the operation to

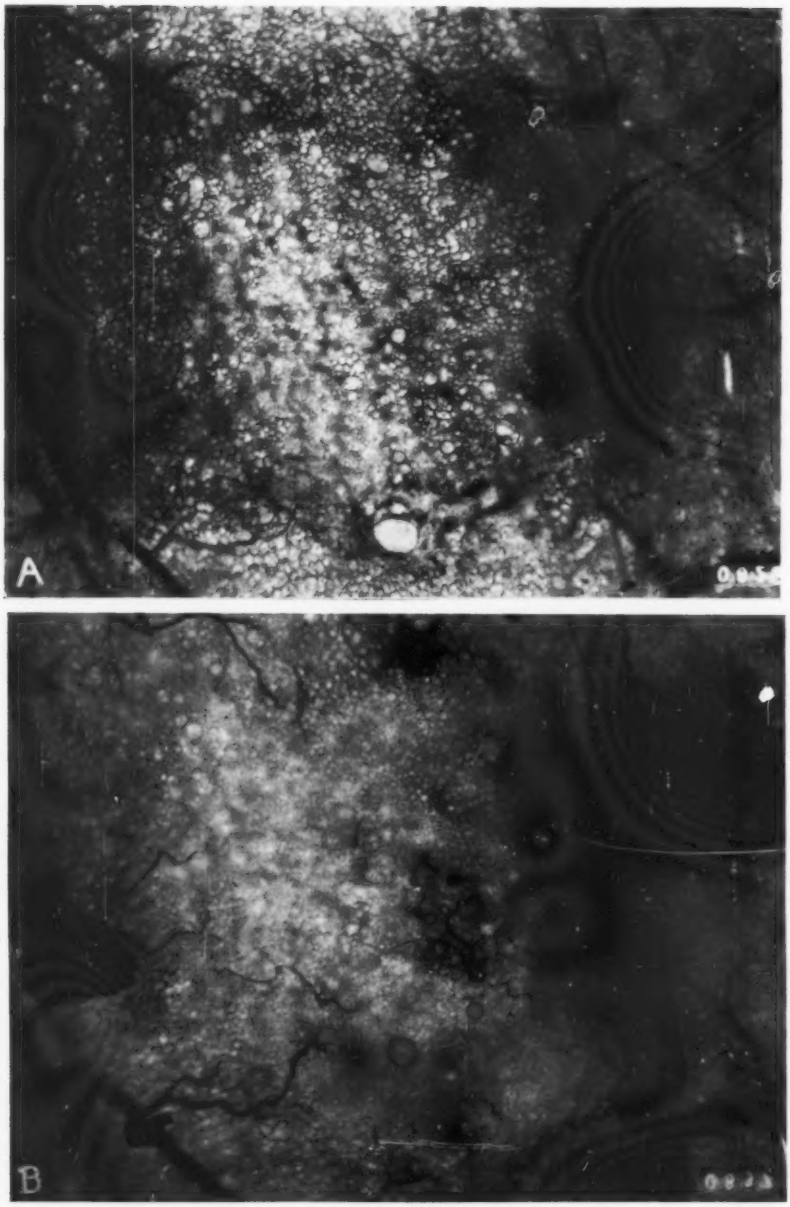

FIGURE 8.—Wound area. *A*, Immediately after operation. Note absence of vessels in the area. *B*, Same area, 6 days later. Note newly developing vascular bed with absence of new vascular tips but showing looped convoluted vessels.

the high-protein diet and maintained thereon for the remainder of the experiment. The curves (fig. 5) show that on the average vascular repair was completed at 9.1 days and that new tips were seen 6.3 days after the operation.

For the fourth experiment, the curves are based on the results of measurements on five mice fed for 24 to 55 days (average 45) prior to operation on the high-protein diet and kept on that diet to the end of the experimental period. These curves (fig. 6) show that the average time of completion of vascular repair was delayed until the sixteenth day (cf. experiments 1, 2, and 3) and for the appearance of new tips until 8.4 days after operation.

The fifth and final experimental series is based on six mice fed the low-protein diet for an average of 25 days before operation and throughout the period after operation. The curves (fig. 7) show that the average time to completion of vascular repair in these animals was also delayed, being 14.7 days, and that new tips were not seen until 7.7 days after operation.

As previously noted and shown on the figures, there was apparent in all experiments a very definite increase in the vascular bed in all wounds prior to the appearance of new vascular tips. It was noted that while definite new vascular tips were not seen, looped or convoluted vessels did make their appearance in the wound area (fig. 8). Their significance is dealt with in the discussion. Examination of the individual data indicated that there was a correlation between the average percentage of vascular bed thus restored in the several experiments and the time when solidification of the exudate into the wound area took place. This type of rise in vascular bed had been noted occasionally during the course of certain of the experiments, but it was only after the final assembling of all quantitative data that the relation between the factors mentioned became strikingly apparent. The average extent and progress of this type of replacement of vascular bed in the five sets of experimental animals is

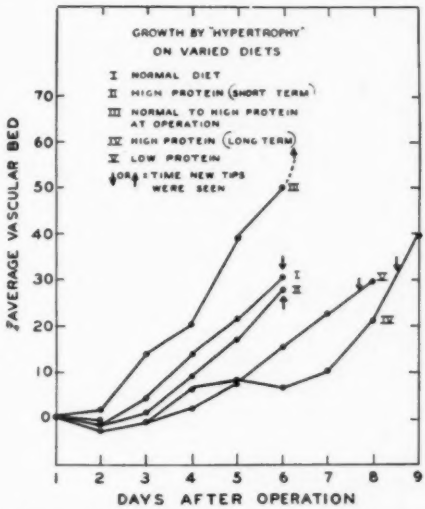

FIGURE 9.—Changes (for all series) in the increase in the vascular bed which resulted from hypertrophy, as defined in the text.

shown in figure 9. The values for the ordinates in these curves represent the volume of the replaced vascular bed (in terms of percent of the average normal vascular bed taken as 100) that is not accounted for by assuming that all inactive vascular tissue left after operation in the wound area was subsequently activated. These curves are all plotted to the day on which new tips were seen.

From this graph and those preceding, the major values pertinent to the evaluation of the data obtained were extracted and are given in table 2.

It is apparent in table 2 that, with the exception of experiment 3, hypertrophy replaced in the neighborhood of 30 percent of the original vascular bed. In experiment 3 it accounted for 49 percent. In experiments 4 and 5 clotting of the exudate, the appearance of new tips, and complete restoration of vascular bed to the average level in the surrounding undamaged tissue was greatly delayed. There is evident a division of the results into two groups: those for experiments 1, 2, and 3, as contrasted with those for experiments 4 and 5.

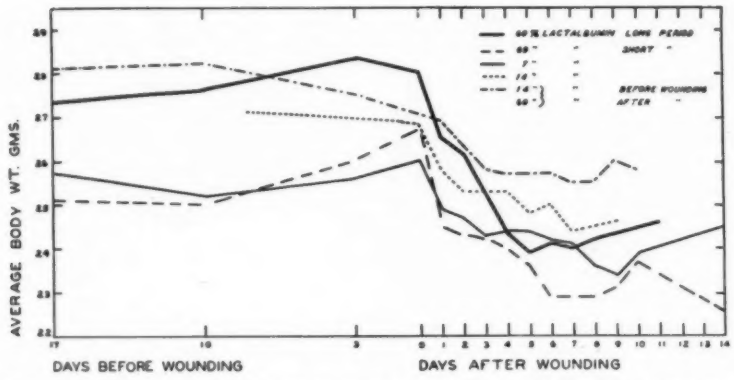

FIGURE 10.—Changes in average weights in mice on the different diets during the experiments.

TABLE 2.—*Effects of level of dietary protein on vascular repair*

Experiment No.	Diet	Day clotting of exudate began	Day exudate completely clotted	Day new tips seen	Increase by hypertrophy	Day of complete vascular repair	
						Average	Range
					Pct.		
1	Normal	7.3	9	6	30	9.8	9–10
2	High protein for short term	7.0	8	6	27	9.4	8–12
3	Normal changed to high at wounding	5.7	7.9	6.3	49	9.1	7–11
4	High protein for long term	[1]>14.4	>14.8	8.4	28	16.0	[2]12–20
5	Low protein	10.7	>14.2	7.7	28	14.7	[2]11–20

[1] In experiments where the sign > is used, most of the wounds of the animals did not clot during the experiments.
[2] In some wounds, the vascular levels did not reach normal during the experiment. These were therefore indeterminate as to healing time but were taken as healed at 20 days for calculation.

The changes in average weights throughout the experimental period in each of the groups is shown in figure 10. There was no significant fluctuation in weights before operation in any experiment. Following operation, in all there was a rapid loss of weight that persisted from 2 to 6 days. This loss was probably the result of operational shock in wounding. Animals showing the least loss of weight after operation (experiments 1 and 3) had on the average a short healing time; those showing a greater loss of weight after operation (experiments 4 and 5) had a greatly delayed healing; the mice in experiment 2 showed the greatest loss in weight but a healing time not significantly longer than that of the animals in experiments 1 and 3. It therefore appears that little if any correlation existed between the weight fluctuations after operation and the time for vascular repair of the wounds.

DISCUSSION

The method of measurement of the developing vascular bed has been thoroughly tested (7) and can be relied upon to give results reproducible within 5 percent. The curves of regrowth of the bed area are sigmoid, as would be expected, in general with the average level of the bed in the surrounding undamaged tissue as the upper asymptote. A test of fit to a logistic curve gives a fairly linear relation for the normal curves; but as before stated, analyses of this sort were not made, since the number of animals seemed too small.

The clean-cut differences, shown between the animals of experiments 4 and 5, as compared with those of experiments 1, 2, and 3 are considered to be highly significant and appear so on "t" test for difference in means. These differences were evident in respect to time of clotting of exudate, time of appearance of new tips, and time of complete vascular repair. The results on time

of vascular repair are also in agreement with the observations of Morris, Dubnik, and Dunn (*9*) on the effect of the level of protein intake on the healing time of wounds in the rat. Inspection of the growth curves also shows differences in rate of vascular repair, as evidenced by the general form of the curves. In all respects the animals of experiments 4 and 5 showed marked delay. It would therefore seem that both a low-protein diet (experiment 4) and a high-protein diet continued for a relatively long period (experiment 5) exert a deleterious effect on the healing process insofar as the restoration of a normal vascular bed is concerned.

The shortest average time for restoration of the bed was that found for experiment 3. While the difference in this experiment is not sufficient to show a conclusive advantage, the fact that the clotting of the exudate occurred earliest and that the animals in this experiment showed the greatest percentage restoration of the bed before new-tip formation may indicate that the slight betterment shown in the values obtained is real and not merely a sampling error.

Perhaps the most interesting finding is the thoroughly well established occurrence common to all animals, of the partial restoration of the vascular bed prior to the new vascular tip formation in the wound area. From table 2 it appears that as much as 49 percent of the new bed may be thus restored.

The manner in which this restoration is accomplished is not perfectly clear, but observation indicates that probably at least three factors contribute: (1) Some part of the damaged vessels rendered static by injury due to the operation regains an active role; (2) dilation of these reactivated vessels serves to increase the volume of the bed; and (3) there appears to be an increase in the length of these and possibly also of relatively uninjured vessels into the wound area, as shown in figure 8, *A* and *B*. This last-mentioned process has, as far as the writers are aware, escaped notice hitherto. To what extent proliferation leading to the intercalation of young cells into the walls of pre-existing vessels is involved is not clear, but that such activity may well play a part in this development seems a reasonable supposition. If this be so, if proliferation occurred, why did not tip formation take place earlier? Allowing for full reactivation of damaged vessels, further proliferation of endothelium sufficient to restore some 30 percent of the original level of the vascular bed appears to have taken place before new tips were formed. How can this be explained?

We suggest that the data in table 2 on the clotting of the exudate in the wound area may furnish ground for an explanation.

It is well-established that cells (for instance, in tissue culture) do not move out from a cell mass, a procedure that would be essential to tip formation, unless a solid substrate is available. Prior to gelation by the laying down of a fibrin clot, such activity (i. e., the formation of endothelial strands) would be prevented. It seemed to us in considering the data that if these conceptions were sound, some correlation would appear between the time at which clotting in the wound area became evident and the time at which new tips were first seen. It is apparent in table 2 that delayed clotting and delay in tip formation go hand in hand. It may also be pointed out that at least 28 percent of the restored bed was formed in all types of wounds (whether slow- or fast-healing) before tips appeared. If this level is essential or connected with clotting (fibrin formation), then the suggestion seems in order that any treatment that would hasten fibrin deposition in the wound area is likely to hasten healing. It may be that stimulation of proliferative activity is probably of little benefit unless coupled with the furnishing of a fibrin network necessary for cell migration.

SUMMARY

Methods for the quantitative study in vivo of the regrowth of vascular tissue

in a subdermal wound in mice are presented.

A study in vivo was made of the effect of different dietary levels of protein (lactalbumin) on the rebuilding of the vascular bed in standardized, subdermal, excised wounds in mice. Three diets were used: low (7 percent) protein, "normal" (14 percent) protein, and high (60 percent) protein.

It was found that both a low-protein diet and a high-protein diet (if the latter was administered for a relatively long period before wounding) greatly delayed vascularization, as compared with the time needed when a normal diet was fed.

A change from normal to high-protein diet or the feeding of a high-protein diet for a short period (15 days) before wounding did not delay vascularization and is possibly beneficial. In all cases a large part (approximately 30 to 49 percent) of the vascular bed was restored in the wound area before the appearance of new vascular tips. The possible mechanism of this type of rebuilding is discussed.

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QUANTITATIVE STUDIES ON THE LATENT PERIOD OF TUMORS INDUCED WITH SUBCUTANEOUS INJECTIONS OF THE AGENT OF CHICKEN TUMOR I. II. ESTIMATION OF THE LATENT PERIOD

By W. Ray Bryan, *senior biologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The latent period of tumors induced with subcutaneous injections of the agent of chicken tumor I (Rous (*1*)) is a function of dose and may therefore be used for bioassay of the tumor agent or for studying host reactions to it. The relationship between latent period per se and dose is nonlinear, and there is a high correlation between the mean and the standard deviation; it is necessary, therefore, to rectify the data, if modern, simplified, statistical methods (*2*, *3*) are to be employed in their analysis. In an earlier paper (*4*), it was shown that conversion of the latent periods to their reciprocals and the dosages to logarithms constitutes a satisfactory transformation of the data for this purpose.

The present report describes a method for estimating the latent periods, based upon practical laboratory considerations and taking into account the changing relationship between latent period and its reciprocal throughout the course of the observation period.

LATENT-PERIOD RESPONSE

The development of a tumor to the point where it can be detected grossly by palpation or by inspection is a gradual process, and the actual time of its origin cannot be determined precisely. Latent-period values given for tumors usually represent the time following inoculation at which they are first recorded during the course of routine periodic examination. The time of the first positive recording is not accepted as an estimate of the latent period unless subsequent recordings on the lesion show its progressive growth; this is the evidence upon which the gross diagnosis of neoplasia has been based. Tumorlike nodules which do not grow progressively should be examined histologically before being accepted as tumors. A more representative value of the actual time of origin of a tumor, if this were of importance, would be the mid-point of the interval between the last negative recording and the first positive recording. Since the value obtained with this and the foregoing procedure would differ by a constant, the variability of the estimated latent periods and their value as indicators of biological activity would be the same in both instances. If the interval between successive examinations changes during the course of the observation period, the time of the first positive recording does not represent so reliable a criterion of biological activity as does the mid-point of the interval between the last negative and the first positive recording. The mid-point of the examination interval is used in the method described herein since the interval is not constant throughout the observation period.

SCHEDULE OF PERIODIC EXAMINATIONS

In the earlier study of the relationship between dose and response (*4*), routine examinations were made at intervals of 1 day; that is, they were spaced equally on a straight time scale. In view of the finding that the reciprocal-of-time is the preferred function for the purpose of statistical analysis, it would be more desirable to have the periodic examinations spaced at equal intervals on a reciprocal-of-time scale; this procedure, however, is not entirely practical. From the relationship between time and its reciprocal (fig. 1 and middle scales of fig. 3), it is seen that a unit increment on the reciprocal scale corresponds to only a few hours during the early course of the observation

period [1] whereas toward the end of the period it represents a time interval of several days. In order to have a constant reciprocal interval which would yield the desired accuracy for late-appearing tumors, it would be necessary to examine the animals every few hours during the early course of an experiment. Such frequent examinations are impractical for two reasons: (1) With the large number of animals required for quantitative experiments, several hours might be required for the completion of one routine examination; (2) the appearance of a tumor is not a sufficiently sharp reaction to permit detection of significant differences within the short space of a few hours, and no increase in final accuracy would be obtained by carrying out routine examinations more than once (or possibly twice in the case of very high concentrations which cause a massive response) on any given day.

Since it is impractical to maintain a constant reciprocal-of-time interval during the first several days of an experiment, the most logical examination procedure for this period seems to be that employed in earlier studies (*4*), namely, examination at 1-day intervals. Just how long daily examinations should be continued in order to obtain the maximum accuracy has not been definitely determined; but it is obvious from inspection of figure 1 that they need not be continued throughout the entire observation period. Toward the end of the period, an interval of 1 day has no more influence on the final results than an interval of 1 or 2 hours would have at the beginning. The curve relating time and its reciprocal (fig. 1) flattens out rapidly after about the tenth day, and this point has been chosen arbitrarily as the limit for daily

[1] The observation period extends from the third or fourth to the thirty-fifth day following inoculation. No tumors have ever been observed as early as the third day, but they are occasionally found on the fourth day when very high doses of the agent have been used. The frequency of tumors that appear after the thirty-fifth day in the test animal population under study (*4*) is so low that it may be neglected; and any site at which a tumor has not developed by this time may be considered as negative.

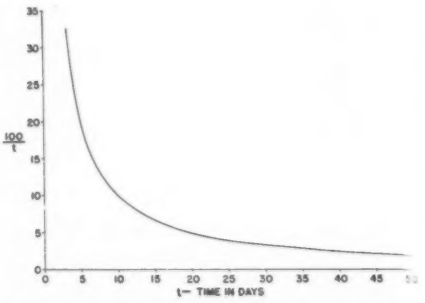

FIGURE 1.—Relationship between time, in days, and its reciprocal (x 100) from the third to the fiftieth day of the observation period.

examinations. After this time, the examinations are spaced as nearly equally as practical on a reciprocal-of-time scale. A provisional examination schedule based upon this procedure is shown in table 1. This schedule is presented as an approximation only, since it is sometimes desirable to alter it slightly to avoid examinations on Sundays and holidays (i. e., after the eighth to tenth day of an experiment), or to permit the overlapping of the schedules of different experiments without conflict of their examination days.

TABLE 1.—*Schedule of routine examinations for quantitative estimation of the latent period*

Successive examination	Time following inoculation		Mean of reciprocals (×100) of interval limits (−) and (+). $\frac{Y, \text{ or } 100}{\text{Latent period}}$	Latent period (harmonic mean of interval) or $\frac{100}{Y}$
	Last negative examination (−)	First positive examination (+)		
	Days	*Days*	$\frac{100}{Days}$	*Days*
1	3	4	29.17	3.43
2	4	5	22.50	4.44
3	5	6	18.33	5.46
4	6	7	15.48	6.46
5	7	8	13.39	7.47
6	8	9	11.81	8.47
7	9	10	10.56	9.47
8	10	12	9.17	10.91
9	12	14	7.74	12.92
10	14	17	6.51	15.36
11	17	21	5.32	18.80
12	21	28	4.17	23.98
13	28	[1] 42	2.98	33.56
14	[1] 42			

[1] See text, footnote 1.

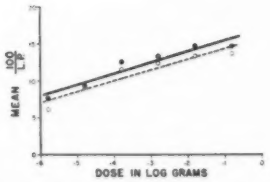

FIGURE 2.—Comparison of results obtained with examinations at 1-day intervals (open circles and broken line) and with the examination schedule of table 1 (approximate) (closed circles and solid line).

Experiments carried out with approximately the same examination schedule as that shown in table 1 gave essentially the same results as those previously published (*4*); the relationship between mean reciprocal and logarithm of dose remained linear, and there was no appreciable difference in the over-all variability of the results of experiments carried out under laboratory conditions otherwise similar. Figure 2 and table 2 show some results obtained by means of the present examination schedule (table 1) in comparison with those previously obtained on a schedule of daily examinations. The recorded data were reanalyzed for the present purpose. The difference between the relative positions of the two curves of figure 2, as well as between the experimental mean responses (Y's) of table 2, was to be expected since the interval limits were taken as estimates of the latent periods in one case while in the other the interval means were used. Such differences are simply a function of the experimental procedure, however, and do not contribute to the variability of results so long as the experimental procedure is constant. The slopes of the dose-response curves obtained by the two examination procedures are practically identical, and the differences between the other statistics (table 2) are no greater than those obtained among the different experiments of a series (*4*) carried out with a common examination technique. The differences between compared variances in the first two dose-group columns (table 2) were due almost entirely to a shift from the interval limit to the interval mean in estimating the latent period; most of the tumors had appeared before the tenth day in these dose groups, and the interval between examinations (1 day) was the same for both schedules up to this time.

The chief advantage gained by use of the examination schedule in table 1 lies, not in the expectation of a greater inherent precision of the biological results, but in the considerable saving in both time and labor, which it makes possible by the elimination of unneces-

TABLE 2.—*Comparison of data obtained with the examinations at 1-day intervals throughout the observation period and the examination schedule of table 1*

Schedule of examination	Dose group (log grams per inoculum)								Homogeneity of variations about dose group means			Experiment mean dose ($\bar{x}$)	Experiment mean response ($\bar{Y}$)	Slope of regression line (b)	Variation about regression line	
	−0.799	−1.799	−2.799	−3.799	−4.799	−5.799	Average	Total	χ^2	Degrees of freedom	P				Variance (s^2)	Degrees of freedom
1-Day intervals									8.97	5	0.12	−3.286	11.02	1.46	4.72	4
Variance of chickens about dose-group means	11.6	6.90	1.47	0.53	1.57	9.20	5.21									
Degrees of freedom	3	3	3	3	3	3		18								
Schedule of table 1 (approximate)									7.13	5	.20	−3.299	12.04	1.49	3.64	4
Variance of chickens about dose-group means	17.4	10.7	2.58	1.72	1.43	3.49	6.22									
Degree of freedom	3	3	3	3	3	3		18								

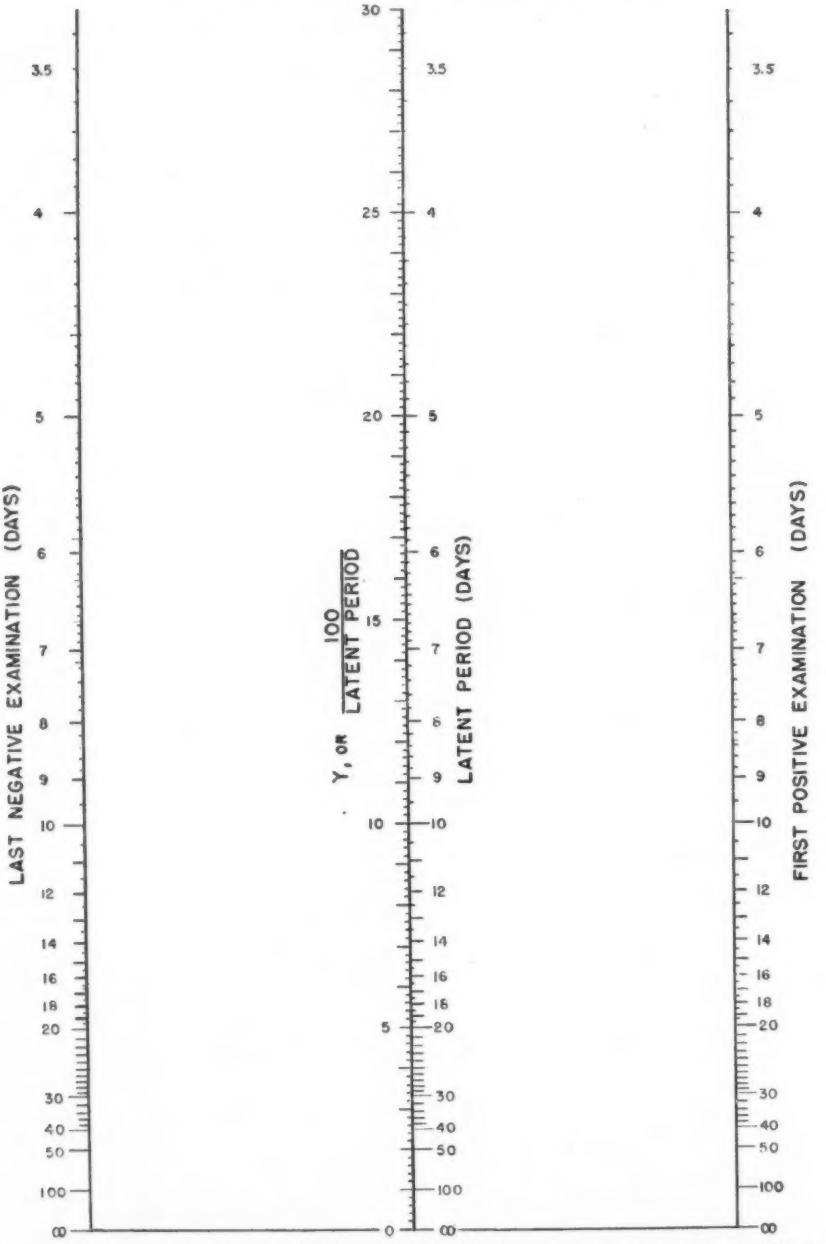

FIGURE 3.—Alignment chart for estimating the latent period and its reciprocal (× 100) from the recorded results of routine periodic examinations. (See text.)

sary and unprofitable routine examinations.

ALIGNMENT CHART FOR ESTIMATING LATENT PERIODS AND THEIR RECIPROCALS (×100)

An alignment chart which facilitates the estimation of latent periods and their reciprocals (×100) is given in figure 3. A straight edge connecting the appropriate interval limits given along the left and right outside scales will cut the scales along the middle line at a point which indicates the corresponding latent period (right) and reciprocal (×100) (left).

SUMMARY

A method is presented for the quantitative estimation of latent periods of tumors induced with the agent of chicken tumor I. Technical and biological limitations, as well as theoretically desirable procedures, were taken into consideration in planning a suggested schedule of routine periodic examinations for determining the time of appearance of tumors. An alignment chart is given which facilitates the determination of latent periods and their reciprocals (which are used for the purpose of statistical analysis).

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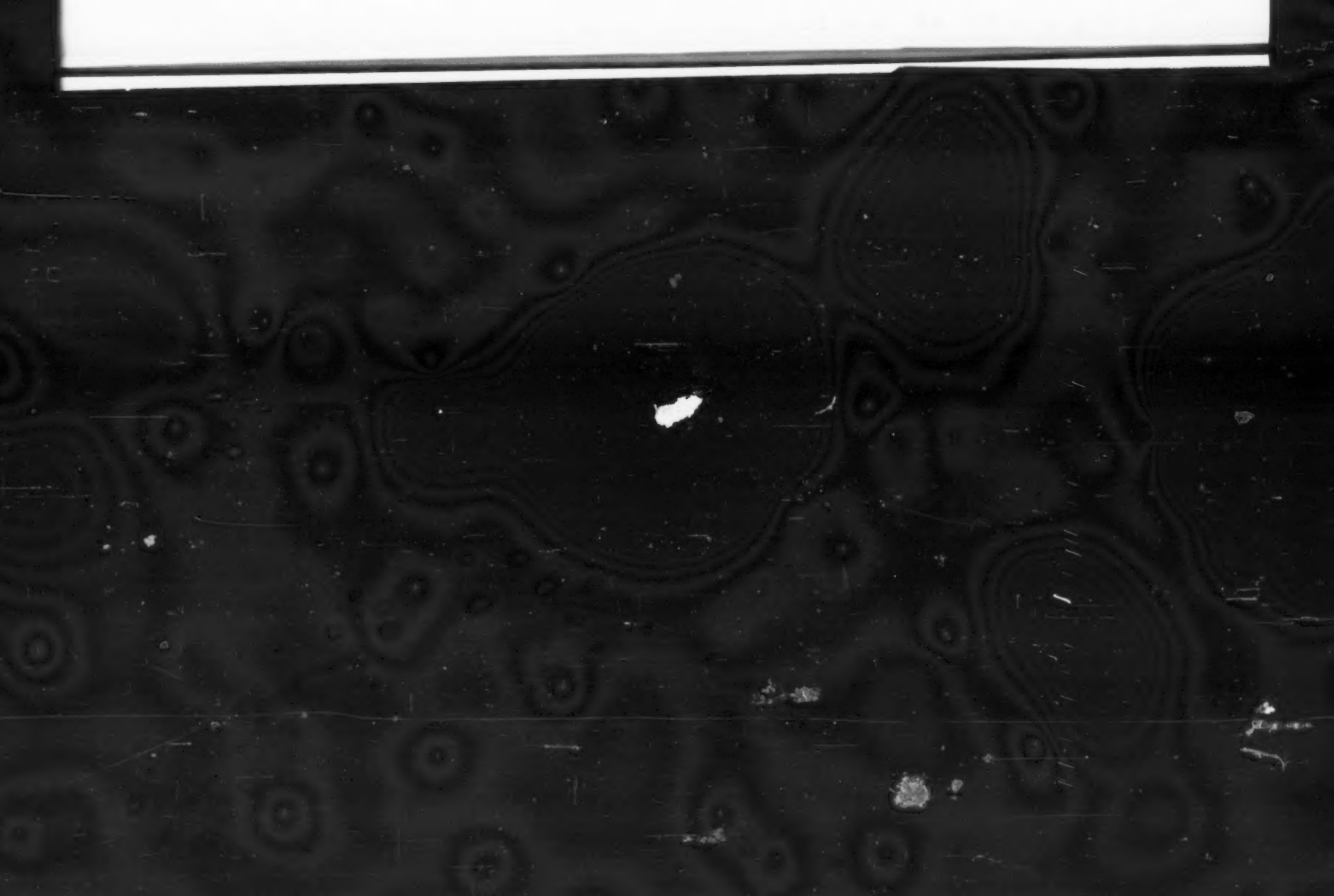

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